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Enzymes of the mandelate pathway in
Acinetobacter calcoaceticus

by

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Thesis submitted to
The University of Glasgow
for the degree of
Doctor of Philosophy

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Abbreviations

Abbreviations used are those recommended in the Biochemical Journal, Instructions to Authors, 1985, with the following additions:

anti-DMDH	- antiserum raised against D-mandelate dehydrogenase
anti-LMDH	- antiserum raised against L-mandelate dehydrogenase
anti-PGDC	- antiserum raised against phenylglyoxylate decarboxylase
BADH	- benzyl alcohol dehydrogenase
BDH	- benzaldehyde dehydrogenase
BSA	- bovine serum albumin
CO	- catechol-1,2-dioxygenase
DAHP	- 3-deoxy- <u>arabino</u> -heptulosonic acid 7-phosphate
DCIP	- 2,6-dichloroindophenol
DLDH	- D-lactate dehydrogenase
DMDH	- D-mandelate dehydrogenase
DMPI	- dimethylpimelimidate
DMSI	- dimethylsuberimidate
DTT	- dithiothreitol
fplc	- fast protein liquid chromatography
GS	- glutamine synthetase
HA	- hydroxyapatite
HRP	- horse radish peroxidase
ICDH	- isocitrate dehydrogenase
KDO	- 3-deoxy-2-keto-octonic acid
LLDH	- L-lactate dehydrogenase
LMDH	- L-mandelate dehydrogenase
MES	- 2- [N-morpholino] ethane sulphonic acid
M_r	- relative molecular mass
MTL	- microtubular lattice
PAGE	- polyacrylamide gel electrophoresis
PDM	- phenylene dimaleimide

PEG	- polyethylene glycol
PEP	- phosphoenolpyruvate
PES	- phenazine ethosulphate
PGDC	- phenylglyoxylate decarboxylase
PMS	- phenazine methosulphate
R	- regulon
SDS	- sodium dodecylsulphate
TCA	- trichloroacetic acid
TEMED	- N,N,N'N'-tetramethylenediamine
TNM buffer, pH8.0	- 0.05M Triethanolamine.HCl buffer, pH8.0 containing 0.1M NaCl and 0.01M MgCl ₂
TPP	- thiamin pyrophosphate (cocarboxylase)
Tween 20	- polyoxyethylene sorbitan monolaurate

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Summary

1. The majority of the work in this thesis was concerned with investigating whether or not the enzymes of the mandelate pathway in Acinetobacter calcoaceticus exist as an enzyme complex. The possible physiological importance of metabolic compartmentation by 'soluble' enzyme interactions in both prokaryotes and eukaryotes is discussed and some examples of such interactions are illustrated in the Introduction.
2. A gentle method for disrupting A.calcoaceticus after lysozyme treatment was developed. The outer and inner membranes of A.calcoaceticus were separated by centrifugation and their purity was assessed by enzyme distribution studies, measurement of KDO, SDS PAGE and electron microscopy. The lactate and mandelate dehydrogenases were found in the inner membrane.
3. A method for the purification of L-mandelate dehydrogenase was developed. This involved Triton X-100 extraction from a 'wall + membrane' fraction, ion exchange chromatography on DEAE-Sephacel, $(\text{NH}_4)_2\text{SO}_4$ fractionation, gel filtration chromatography on Ultrogel AcA 34 followed by a second ion exchange on DEAE-Sephacel. L-Mandelate dehydrogenase was purified almost to homogeneity as determined by SDS PAGE.
4. Antibodies were raised against the denatured enzyme by cutting the protein-stained band from an SDS polyacrylamide gel. Although the antibodies had no effect on enzyme activity, a positive band corresponding to L-mandelate dehydrogenase was detected on immunoblotting bacterial extracts containing L-mandelate dehydrogenase activity. No such band was observed on immunoblotting extracts from mutants with no detectable L-mandelate dehydrogenase activity (C1123 and C1408).
5. Some properties of the purified L-mandelate dehydrogenase were determined to allow a comparison with those of the purified D-mandelate dehydrogenase and lactate dehydrogenases from A.calcoaceticus and the lactate dehydrogenases from other bacteria. L-Mandelate dehydrogenase was found to be a monomer with an M_r of 44,000, pH optimum of 7.5 and

K_m for L-mandelate of 186 μ M. L-Mandelate dehydrogenase was shown to be completely stereospecific for its substrate and neither D nor L-lactate acted as a substrate. L-Mandelate dehydrogenase was inhibited by D-mandelate but only at high concentrations (≥ 10 mM). L-Mandelate dehydrogenase was unaffected by metal chelating reagents but was sensitive to reagents which react with sulphydryl groups such as mercuric chloride and p-chloromercuribenzoate. FMN was identified as the non-covalently bound cofactor of L-mandelate dehydrogenase on the basis of the fluorescence of the acid extractable material.

6. Problems were encountered with the aldehyde dehydrogenase-linked phenylglyoxylate decarboxylase assay when there were high amounts of NADH oxidase activity present in the sample and so a method was developed linking phenylglyoxylate decarboxylase activity to alcohol dehydrogenase activity. This method proved accurate and reliable and it was possible to correct for NADH oxidase activity in the sample by including a minus substrate (phenylglyoxylate) control.

7. Several methods were used to study possible enzyme interactions in the mandelate pathway of A.calcoaceticus.

a) Extracts prepared after disrupting bacteria by sonication or French pressing or after lysozyme treatment were studied by ultracentrifugation. As well as the mandelate enzymes, the control enzymes, catechol-1,2-dioxygenase (a low M_r 'soluble' enzyme from a related part of metabolism), isocitrate dehydrogenase (a low M_r 'soluble' enzyme from an unrelated part of metabolism) and glutamine synthetase (a 'soluble' enzyme from an unrelated part of metabolism with a high $M_r = 600,000$) were assayed in these experiments. Phenylglyoxylate decarboxylase has previously been believed to be a 'soluble' enzyme, however, the amount of phenylglyoxylate decarboxylase recovered in the high speed pellet (where most of the L-mandelate dehydrogenase activity was located) varied (5-55%) with the disruption method whereas more or less the same amount of glutamine synthetase (30-40%) sedimented irrespective of the method of disruption.

It was concluded that the high proportion of phenylglyoxylate decarboxylase recovered in the membrane fraction after bacteria had been disrupted by two passages through the French press was not simply a consequence of the M_r (230,000) of this enzyme. Homogenates prepared by disrupting bacteria by two passages through the French press were used to further investigate the association of phenylglyoxylate decarboxylase with the membrane fraction.

b) The amount of phenylglyoxylate decarboxylase recovered in the pellet varied with the protein concentration of the homogenate. At high protein concentrations (60mg ml^{-1}), 92% phenylglyoxylate decarboxylase was recovered in the pellet whereas at low protein concentrations (3mg ml^{-1}) only 22% phenylglyoxylate decarboxylase was recovered in this fraction. The amount of the control 'soluble' enzymes recovered in the pellet also varied with the protein concentration of the homogenate but there was always substantially more phenylglyoxylate decarboxylase than the other 'soluble' enzymes in this fraction. That so much phenylglyoxylate decarboxylase could be recovered in the high speed pellet was taken to indicate the possibility of a specific association of phenylglyoxylate decarboxylase with the membrane.

c) Particular care was taken in the choice of disruption buffers. However, attempts to remove the non-specifically bound control 'soluble' enzymes from the membrane fraction while leaving PGDC associated were unsuccessful.

d) Homogenates prepared by French press treatment were studied by gel filtration chromatography and density gradient centrifugation. However, no evidence for the co-purification of L-mandelate dehydrogenase and phenylglyoxylate decarboxylase was obtained even in the presence of high protein concentrations.

- e) No evidence of co-immunoprecipitation of the enzymes by anti-D-mandelate dehydrogenase, anti-L-mandelate dehydrogenase or anti-phenylglyoxylate decarboxylase was obtained.
 - f) The in vivo crosslinking experiments with dimethylsuberimide (which crosslinks lysine residues) on Pseudomonas putida reported by Halpin et al. (1981) were repeated and expanded. Control and crosslinked extracts were analysed by ultracentrifugation and gel filtration chromatography but in contrast to the conclusions made by these authors, it was clear that only non-specific crosslinking was occurring.
 - g) No specific crosslinking was observed in similar experiments on A.calcoaceticus even when the bacteria were supplied with substrates and cofactors for the mandelate enzymes.
 - h) It is generally thought that a high pH (> 8.0) is required for dimethylimidate crosslinking activity. However, since phenylglyoxylate decarboxylase dissociates at pH 8.0, a method for crosslinking both intact bacteria and homogenates with these reagents at pH 7.5 was developed.
 - i) Control and crosslinked extracts were analysed by immunoblotting with anti-D-mandelate dehydrogenase, anti-L-mandelate dehydrogenase and anti-phenylglyoxylate decarboxylase. Although there was evidence for the formation of high M_r species, there was no evidence for specific crosslinking of phenylglyoxylate decarboxylase to either of the mandelate dehydrogenases.
 - j) No specific crosslinking with phenylene dimaleimide (which crosslinks sulphydryl groups) was obtained when either intact bacteria or homogenates prepared by French press treatment were treated with phenylene dimaleimide.
8. The difficulties relating to the possible lability of enzyme interactions and the inadequacy of the techniques available for studying such interactions, as well as possible future experiments, are discussed in the final chapter of the thesis.

CHAPTER 1 INTRODUCTION

1.1 Acinetobacter calcoaceticus

Until recently the genus Acinetobacter was generally considered to comprise the single species calcoaceticus which was divided into four biotypes: anitratus, haemolyticus, alcaligenes and lwoffii (Vivian et al., 1981). However, Bouvet & Grimont (1986) have proposed that the genus Acinetobacter be divided into six species: calcoaceticus, lwoffii, haemolyticus, baumannii, junii and johnsonii.

All acinetobacters are aerobic, gram-negative, non-motile (although 'twitching' has been demonstrated in some strains), catalase-positive and oxidase-negative, with a G+C content of 38-47 mole % (Henriksen, 1976). Most strains have an optimum growth temperature of 34-35°C (Breuil et al., 1975).

Probably as a result of their nutritional versatility, acinetobacters have a wide distribution in nature and have been isolated from soil, water and sewage (Baumann 1968; Warskow & Juni, 1972; Henriksen, 1973). Acinetobacters have also been found to reside in or on many parts of the human body, possibly as a result of contamination from the surroundings or as a consequence of the fact that these bacteria have an optimum growth temperature around 35°C (Juni, 1972; Henriksen, 1973; Gaughan et al., 1979). Presumably because of their hydrocarbon degrading properties, acinetobacters have also been found associated with oil pollution in aquatic environments and industrial effluents (Gutnick Rosenberg, 1977; Bartha & Atlas, 1977). Indeed, A.calcoaceticus is of industrial importance since it can produce an extracellular polyanionic emulsifier called 'emulsan' (Rubinovitz et al., 1982).

Recently, Acinetobacter has received increasing attention because of its apparent pathogenic properties. It has been associated with a variety of infections such as septicaemia and urinary tract infections (Glew et al., 1977; Retailliou et al., 1979; Lowes et al., 1980;

Hoffman et al., 1982), endocarditis (Henriksen, 1973; Cohen et al., 1980; Rao et al., 1980), meningitis (Ghoneim & Halaka, 1980; Berk & McCabe, 1981; Kabayashi et al., 1983), pneumonia (Glew et al., 1977; Rudin et al., 1979; Holton & Shorvon, 1982; Markham & Telfor-Brunton, 1983), respiratory tract infections (O'Connell & Hamilton, 1981), skin and wound infections (Glew et al., 1977) and peritonitis (Roxe & Santhanam, 1983; Larson, 1984). However, some evidence suggests that Acinetobacter is merely an opportunistic bacteria and its pathogenicity may be overestimated (Juni, 1972; Markham & Telfor-Brunton, 1983).

Acinetobacters are often resistant to many antibiotics (French et al., 1980; Markham & Telfor-Brunton, 1983; Holton & Shorvon, 1983) and in some cases this has been shown to be due to transferable plasmids (Hinchliffe & Vivian, 1980a; Devaud et al., 1982; Goldstein et al., 1983). This genus is relatively resistant to ionising radiation (Kairiyama et al., 1979) which is sometimes used in food preservation and as a result, acinetobacters have been associated with food spoilage (Ito et al., 1976; Firstenberg-Eden et al., 1980).

The wide nutritional versatility, frequency of occurrence and possible clinical importance of acinetobacters has stimulated research in the development of systems of genetic transformation (Juni, 1978; Ahlquist et al., 1980), transduction (Herman & Juni, 1974; Juni, 1978) and conjugation using plasmids (Towner & Vivian, 1976a,b; Towner, 1978; Hinchliffe & Vivian, 1980a,b,c). In addition, a circular linkage map has been established using plasmid RP4 mediated chromosome transfer (Towner & Vivian, 1976b; Towner, 1978) and the discovery of the involvement of transposition in this event should prove useful in improving this technique (Towner, 1983).

Strain NCIB 8250 has been classified as a strain of Acinetobacter calcoaceticus (Fewson, 1967a,b; Baumann et al., 1968). This organism, which grows at an optimal temperature of 30°C (Fewson, 1967b), is unable to metabolise carbohydrates (Cook & Fewson, 1973) but can utilise a wide variety of organic compounds, including many aromatics, as sole source of carbon and energy (Fewson, 1967b).

1.2 The mandelate pathway and its regulation in *A.calcoaceticus*

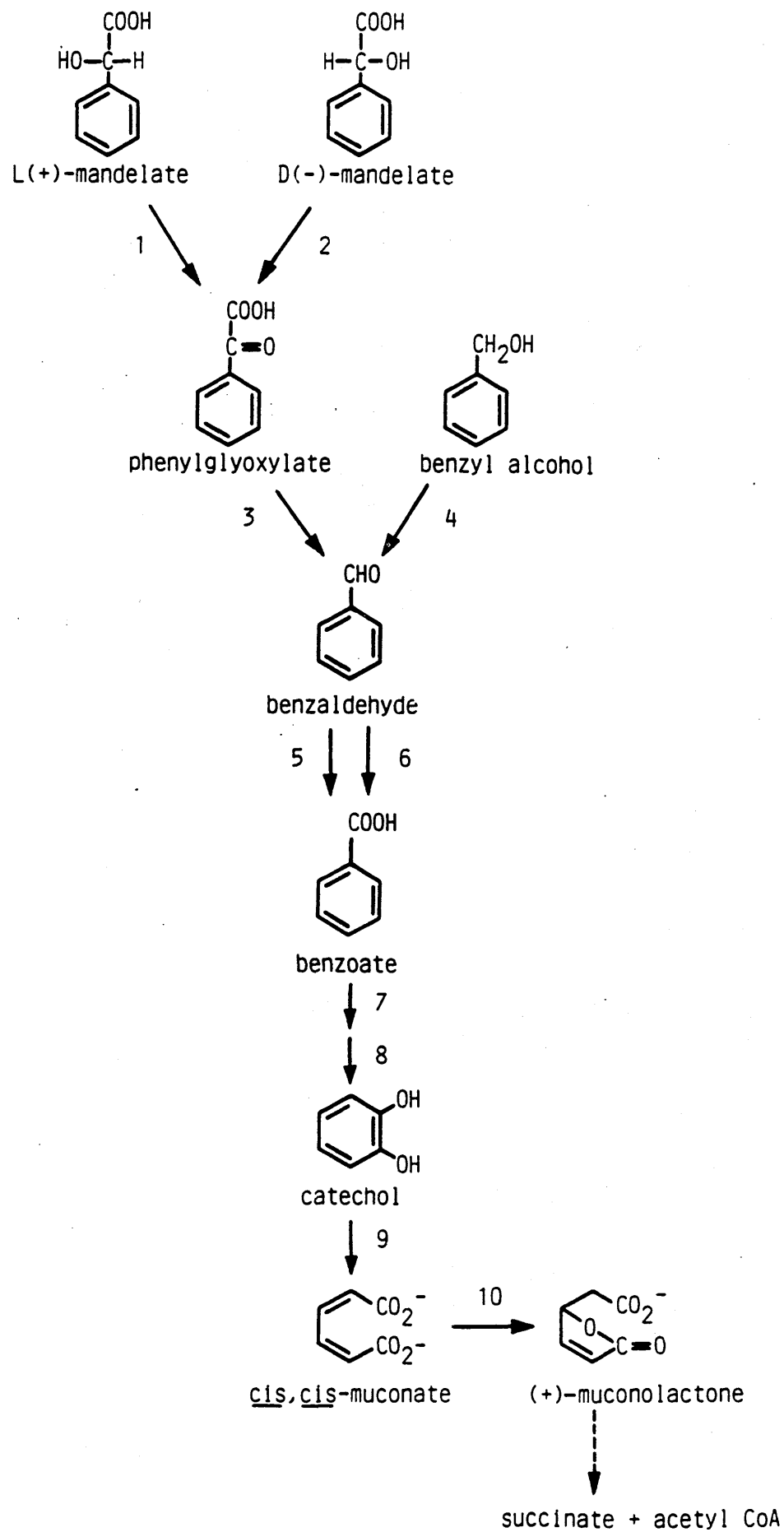
Mandelic acid is an aromatic acid of plant origin (Stanier, 1948) and both D(-) and L(+) isomers occur in nature. Mandelic acid is also found in the urine of industrial workers exposed to styrene (Ohtsuji & Ikeda, 1970; Elia et al., 1980). Several organisms can use mandelate as sole source of carbon and energy; these include bacteria of the genera Pseudomonas (Stainer et al., 1966) and Acinetobacter (Baumann et al., 1968); the fungi Aspergillus niger (Jamaluddin et al., 1970) and Neurospora crassa (Ramakrishna Rao & Vaidyanathan, 1977) and the yeast Rhodotorula graminis (Durham, 1984).

The pathway for the catabolism of mandelate in *A.calcoaceticus* is illustrated in Figure 1. Using the technique of simultaneous adaptation (Stanier, 1947), Kennedy & Fewson (1968a) showed that in *A.calcoaceticus* wild type NCIB 8250, L-mandelate was oxidised to catechol via phenylglyoxylate (benzoyl-formate), benzaldehyde and benzoate. Benzyl alcohol was converted to catechol in a convergent pathway via benzaldehyde. Growth on mandelate or phenylglyoxylate resulted in the appearance of L(+)-mandelate dehydrogenase (LMDH), phenylglyoxylate decarboxylase (PGDC), benzyl alcohol dehydrogenase (BADH) and a heat-stable as well as a heat-labile benzaldehyde dehydrogenase (BDH I and II respectively) activities (Kennedy & Fewson, 1968b). BADH and the heat-labile BDH were found in extracts of bacteria grown on benzyl alcohol or benzaldehyde.

The mandelate pathway in *A.calcoaceticus* can be divided into three separate regulatory units (Figure 1): regulon 1 (R1) comprises LMDH, PGDC and BDH I; regulon 2 (R2) comprises BADH and BDH II; benzoate oxygenase and cyclohexadienediol carboxylate dehydrogenase constitute regulon 3 (R3) (Livingstone & Fewson, 1972). The term 'regulon' relates to a group of genes controlling related functions and regulated together

Figure 1 Metabolism of mandelate and benzylalcohol in A.calcoaceticus

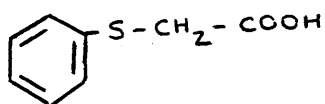
- Regulon 1 : L(+)-mandelate dehydrogenase (1)
D(-)-mandelate dehydrogenase (2)
phenylglyoxylate decarboxylase (3)
benzaldehyde dehydrogenase I (5)
- Regulon 2 : benzylalcohol dehydrogenase (4)
benzaldehyde dehydrogenase II (6)
- Regulon 3 : benzoate oxygenase (7)
cyclohexadienediol carboxylase dehydrogenase (8)
- 'Catechol pathway' enzymes = catechol-1,2-dioxygenase (9)
(+)-muconate lactonising enzyme (10)



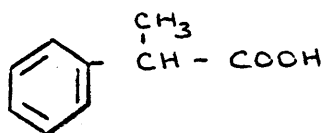
by an inducer or repressor (Maas & Clark, 1964).

LMDH, PGDC and BDH I are coordinately synthesised and are referred to as the 'mandelate enzymes' or R1 enzymes (Livingstone & Fewson, 1972). Phenylglyoxylate, the product of the first enzyme of the pathway (Figure 1) is the primary inducer of R1 and the mandelate enzymes are specifically and gratuitously induced by thiophenoxyacetate (Livingstone & Fewson, 1972). 2-Phenylpropionate is a competitive inhibitor of induction and was used for the isolation of constitutive mutants (Fewson *et al.*, 1978).

THIOPHENOXYACETATE



2-PHENYLPROPIONATE



A.calcoaceticus, wild type NCIB 8250, has a dehydrogenase specific for L-mandelate and can therefore grow on the L isomer of mandelate but not on D-mandelate (Kennedy & Fewson, 1968a,b). However, mutants have been isolated which can grow on D-mandelate (Fewson *et al.*, 1976; Hills & Fewson, 1983a). These mutants contain a novel D(-)-mandelate dehydrogenase (DMDH) in addition to the original LMDH (Hills & Fewson, 1983a). A second wild type strain, EBF 65/65, shows the opposite pattern in that it can grow on D-mandelate but not on L-mandelate and mutants have been isolated from this strain which possess an LMDH as well as the original DMDH and can therefore grow on L-mandelate (Hills & Fewson, 1983a). It was shown that the novel enzymes were coordinately controlled with the pre-existing enzymes, i.e. they form part of R1 (Hills & Fewson, 1983b).

The mandelate enzymes are repressed in the presence of benzoate, catechol and succinate. The latter two compounds also inhibited these enzymes but there is no evidence for feedback inhibition by the other intermediates of the pathway (Cook *et al.*, 1975).

The mandelate pathway converges with the oxidation of benzyl alcohol at the level of benzaldehyde (Cook *et al.*, 1975). The ability of both

benzaldehyde and benzyl alcohol to induce R2 is in contrast with the specificity of phenylglyoxylate as the inducer of the enzymes of the mandelate pathway.

The further metabolism of benzoate to catechol (Figure 1) involves the enzymes benzoate oxygenase and cyclohexadienediol carboxylate dehydrogenase which are often referred to as 'benzoate oxidase' (Reiner, 1971; Reiner & Hegeman, 1971; Reiner, 1972). In NCIB 8250, catechol is converted via cis,cis-muconate and (+)-muconolactone to succinate and acetyl CoA (Figure 1) by the catechol branch of the β -ketoadipate pathway (Kennedy & Fewson, 1968b).

1.3 Properties of the mandelate enzymes in *A.calcoaceticus*

Both LMDH and DMDH have been designated 'membrane-bound' (Hills & Fewson, 1983a) although it is not known whether they are outer or inner membrane enzymes. Experiments with crude extracts showed that LMDH and DMDH are NAD(P) independent, show absolute stereospecificity for their substrates and are inhibited by the opposite isomer (Hills & Fewson, 1983a). Both enzymes have similar dependence on temperature and pH and are inhibited by oxalate but not by several metal chelating agents. DMDH is much more susceptible to inhibition by HgCl_2 and p-chloromercuribenzoate and is much more heat-labile than LMDH (Hills & Fewson, 1983a).

In addition to the stereospecific mandelate dehydrogenases, a pair of stereospecific lactate dehydrogenases can be coordinately induced in *A.calcoaceticus* by either D(-) or L(+)-lactate (Fewson & O'Donnell, 1981; Allison et al., 1985a). All four dehydrogenases, although separable, have been found to be very similar to each other (Fewson & O'Donnell, 1981) e.g. all are membrane-bound and NAD(P) independent. Furthermore, D-lactate dehydrogenase (DLDH) and DMDH are strikingly similar, as are L-lactate dehydrogenase (LLDH) and LMDH (Fewson & O'Donnell, 1981; Allison et al., 1985a). This is illustrated by the heat-lability and sensitivity to inhibition by p-chloromercuribenzoate. The enzymes specific for the D enantiomer of lactate and mandelate are also more easily solubilised from membranes (Fewson & O'Donnell, 1981; Allison et al., 1985a).

None of the dehydrogenases was significantly solubilised by methods commonly used for the removal of peripheral proteins (Allison et al., 1985a). However, all four enzymes were solubilised by detergents, especially Triton X-100 and Lubrol PX. The similarities between the enzymes specific for the D enantiomers of mandelate and lactate was again shown by the selective solubilisation of these two enzymes by the

ionic detergents cholate, deoxycholate and sodium dodecyl sulphate (Allison et al., 1985b). This treatment left LLDH and LMDH associated with the membranes and still in an active form. However, attempts to solubilise these enzymes with Triton X-100 after cholate extraction proved unsuccessful. Allison et al. (1985a,b) described the optimal conditions for the solubilisation of the four enzyme activities. The detergent: protein ratio was especially important since the enzymes were not released if not enough detergent was added and the enzymes were inactivated if too much detergent was added. Maximal solubilisation of DMDH and DLDH from a 'wall + membrane' fraction was achieved with 1.5mg cholate/mg protein and 0.5mg Triton X-100/mg protein was the optimal detergent concentration for solubilising LMDH and LLDH (Allison et al., 1985b).

The four enzyme activities have been separated by DEAE-Sephacel ion exchange chromatography (Fewson & O'Donnell, 1981; Allison et al., 1985b) and a purification scheme for DMDH and DLDH has been developed (Allison et al., 1985b). This purification involved cholate extraction from a 'wall + membrane' fraction, $(\text{NH}_4)_2\text{SO}_4$ fractionation, gel filtration and ion exchange chromatography and chromatofocusing. DLDH and DMDH were co-eluted from the Ultrogel Aca 34 gel filtration column (apparent $M_r = 130,000$). Ion exchange chromatography of the pooled fractions from gel filtration separated the two enzymes, with DLDH eluting at a lower phosphate concentration (70-80mM) than DMDH (100-110mM). The final purification of the two enzymes was achieved by chromatofocusing.

The properties of the purified DMDH and DLDH were shown to be very similar (Allison et al., 1985b). The apparent M_r of DLDH was 62,800 whereas that of DMDH was 59,700. Both enzymes gave similar responses to pH and were sensitive to p-chloromercuribenzoate but metal chelating agents did not affect either activity. In addition, both enzymes were shown to contain non-covalently bound FAD as cofactor (Allison et al.,

1985b).

The second enzyme of the mandelate pathway is PGDC (Figure 1). This enzyme catalyses the thiamin pyrophosphate (TPP) dependent, non-oxidative decarboxylation of phenylglyoxylate to benzaldehyde and remains in the soluble fraction obtained after differential centrifugation of bacterial extracts prepared by ultrasonic treatment (Kennedy & Fewson, 1968b). PGDC is an allosteric enzyme which exhibits negative cooperativity towards the coenzyme and ADP (Jamaluddin & Fewson, 1973). PGDC has been purified to homogeneity (Barrowman & Fewson, 1985) and is a tetrameric enzyme with an apparent subunit M_r of 58,000.

BADH, BDH I and BDH II are NAD^+ dependent and all are 'soluble' enzymes (Kennedy & Fewson, 1968b). Experiments with crude extracts showed that BDH I and BDH II were shown to be sensitive to p-chloromercuribenzoate and N-ethylmaleimide and optimal activities were achieved at pH 9.0-9.5 (Livingstone et al., 1972). BADH and BDH II have been purified to homogeneity and both are tetrameric enzymes with subunit M_r values of 39,700 and 55,000 respectively (MacKintosh, 1986; MacKintosh & Fewson, 1986). A purification scheme is currently being developed for BDH I which is also tetrameric with a subunit M_r of 55,000 (personal communication, R.M. Chalmers).

1.4 Mandelate metabolism in Pseudomonas putida

P.putida strain NCIB 9494, previously referred to as P.fluorescens A.3.12, has been shown to metabolise L-mandelate through the same sequence of metabolic intermediates as A.calcoaceticus (Gunsalus et al., 1953a,b; Hegeman, 1966a,b,c; Ornston & Stanier, 1966; Cánovas et al., 1967). In addition, P.putida, strain NCIB 9494, converts D-mandelate to L-mandelate by means of a racemase (Gunsalus et al., 1953a,b; Hegeman et al., 1970; Hegeman & Kenyon, 1970; Maggio et al., 1975; Whitman et al., 1982), although Hegeman et al. (1970) have described another strain of P.putida which has a DMDH but has no racemase. As in A.calcoaceticus the LMDH of P.putida is membrane-bound (Gunsalus et al., 1953a,b); however, this enzyme has not been solubilised or purified.

P.putida synthesises three benzaldehyde dehydrogenases : one is NAD⁺-linked, one is NADP⁺-linked (Gunsalus et al., 1953a,b) and the third is synthesised during growth on benzaldehyde (Stevenson & Mandelstam, 1965). Metabolism of benzyl alcohol also seems to be via dehydrogenation to benzaldehyde as in A.calcoaceticus (Hegeman, 1966a) however, P.putida oxidises benzyl alcohol slowly by non-specific alcohol and aldehyde dehydrogenases which do not normally function in mandelate metabolism (Colins & Hegeman, 1984).

The mandelate pathway in P.putida is controlled by induction and repression as in A.calcoaceticus. However, R1 is non-specifically induced by either L-mandelate, D-mandelate or phenylglyoxylate (Stanier et al., 1964; Hegeman, 1966a,b,c; Higgins & Mandelstam, 1972). Repression is by benzoate, catechol and succinate (Mandelstam & Jacoby, 1965; Stevenson & Mandelstam, 1965); since the repression mechanism can be activated independently by any one of these repressors, it was called a 'multisensitive repression mechanism'.

1.5 Enzyme interactions

1.5.1 Metabolic compartmentation in prokaryotes and eukaryotes

Prokaryotes (cells without distinct nuclei) differ from eukaryotes (cells with nuclei separated from the cytoplasm by a membrane) in many respects but one striking difference is in the level of subcellular organisation. Eukaryotes contain subcellular organelles e.g. mitochondria and/or chloroplasts which effectively segregate enzymes and metabolites within the cell and the importance of this compartmentation in the regulation of cellular processes is well established (Srere & Estabrook, 1978; Nover et al., 1980; Friedrich, 1984, 1985). Further compartmentation is also achieved in the eukaryotic cytoplasm by the presence of multifunctional proteins e.g. the arom complex and fatty acid synthetase (Kirschner & Bisswanger 1976) and by the presence of stable multienzyme complexes e.g. pyruvate dehydrogenase, α -ketoglutarate dehydrogenase and tryptophan synthase (Welch, 1977; Hammes, 1981). Although intracytoplasmic membranes have been observed in bacteria (Cota-Robles, 1966; Weigand et al., 1973; Kennedy & Finnerty, 1975) there are no subcellular organelles present and consequently bacteria lack the metabolic compartmentation such organelles confer on cells.

The degree of organisation of the prokaryotic cytoplasm is unknown but multifunctional proteins (Kirschner & Bisswanger, 1976) and stable multienzyme complexes similar to those of eukaryotic systems have been isolated from bacteria e.g. pyruvate dehydrogenase from E.coli (Reed & Cox, 1970) and from Bacillus stearothermophilus (Packman & Perham, 1982) and tryptophan synthase from E.coli (Miles, 1979). The enzymes of fatty acid synthesis are usually found as individual activities in bacteria but a single polypeptide chain with complete fatty acid synthesis activity has been isolated from Mycobacterium smegmatis (Wood et al., 1978). The fatty acid β oxidising (degradative) enzymes have been

isolated as a stable five enzyme cluster from E.coli and other bacteria (Ottaway & Mowbray, 1977).

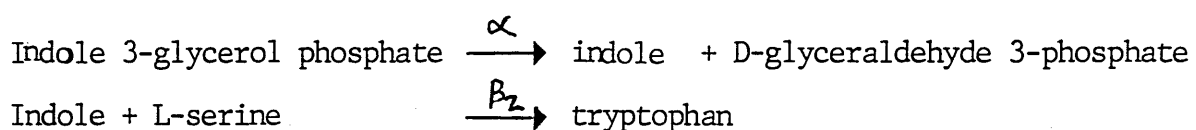
There is also the possibility that metabolic compartmentation could be achieved in prokaryotes, and in eukaryotes, by the association of apparently 'soluble' sequential enzymes of a metabolic pathway in the form of transient enzyme complexes (Srere & Mosbach, 1974; Friedrich, 1984, 1985). Friedrich (1984) has outlined a model for 'dynamic compartmentation' involving such transient enzyme-enzyme complexes. In this model, consecutive enzymes may accomplish direct metabolite transfer (channeling) even if the complexes are very loose and the rate constants for both association and dissociation are high. The non-random distribution of enzymes in macrocompartments would favour collisions between functionally adjacent enzymes and hence augment the compartmenting effect. The flexibility of association of 'soluble' enzymes to meet the changing requirements of the cytoplasm could theoretically have greater advantage to the cell than other kinds of spatial compartmentation. There is also the possibility that 'soluble' sequential enzymes associate weakly but not necessarily transiently.

1.5.2 Advantages of enzyme complexes

The organisation of enzymes which catalyse sequential reactions in stable or transient complexes could confer several advantages on the cell (Welch, 1977; Hammes, 1981; Friedrich, 1984).

a) Enhancement of catalytic activity

Enhancement of catalytic activity could be brought about as a result of stabilisation of the active conformational state of each component enzyme. This is illustrated by tryptophan synthase of E.coli which consists of two polypeptide chains and the enzyme complex has the structure of $\alpha_2\beta_2$. Separately the α and β subunits have distinct activities :



However, the overall activity of the complex ($\alpha_2\beta_2$) is 30-100 fold greater than the individual subunits (Creighton, 1970; Ginsberg & Stadtman, 1970). Furthermore, mutants have been isolated with inactive α and β subunits in that they cannot catalyse their respective reactions but they have been shown to activate the complementary subunit, which suggests that a conformational change is induced (Yanofsky & Crawford, 1972; Miles, 1979).

Tryptophan synthase is one of the few good examples of complexes which show enhanced catalytic activity. The presence of unforeseen enzyme effectors in a multicomponent reaction could complicate the interpretation of experiments designed to illustrate enhanced catalytic activity due to enzyme-complex formation. Therefore, experiments which purport to demonstrate that the rate of a multistep sequence of reactions proceeds more rapidly than would be expected on the basis of the summation of the individual enzyme-catalysed reaction velocities should be regarded with some caution. Ottaway (1985) reinforced this need for scepticism when in a computer simulation he was unable to show a change in flux through a coupled enzyme system. This study involved a comparison of the reaction velocities of malate dehydrogenase and citrate synthase free in solution with that of the enzyme pair packed closely together in a particle and of the enzymes immobilised on an inert matrix (Ottaway, 1984, 1985).

b) Substrate channeling

The slow dissociation of an intermediate produced by one enzyme in a sequence could provide a high steady state concentration for the next enzyme and by keeping an intermediate in a limited environment, competition with other pathways could be minimised. Tryptophan synthase again provides a good example of this; indole, the product of the α subunit reaction, cannot be detected as a free intermediate of the tryptophan synthase complex (Matchett, 1973).

Evidence for substrate channeling in general, however, has been difficult to achieve; indeed Coggins & Boocock (1986) have been unable to find any direct evidence of substrate channeling in the arom complex of N.crassa from measurements of steady state levels of several intermediates. Indirect evidence for channeling in other systems comes from in vivo isotope tracer experiments (see 1.5.5.3; Shipley & Clark, 1972; Bhargava, 1985) but these are often limited by the resolution of the techniques used to detect labelled intermediates and by a limited knowledge of the effectors controlling a particular pathway. Furthermore, exogenously applied intermediary metabolites may be inaccessible to enzyme sites because of permeability barriers or they may be metabolised before they come in contact with the enzyme system under study.

c) Unstable or toxic intermediates

A suitable environment could be provided for unstable intermediates thereby protecting them from uncontrolled non-enzymic reactions e.g. decarboxylation, hydrolysis, hydration or oxidation. Furthermore, localisation of toxic intermediates would minimise their detrimental effects on the cell.

d) Decrease in transit and in transient time

The transit time is the time required for a metabolite to reach the next enzyme in a pathway and the transient time is the parameter which characterises the speed of attaining a (new) steady state (Welch, 1977; Gaertner, 1978). The presence of enzyme complexes could decrease both the transit and the transient time as a result of direct metabolite transfer from one enzyme to another. This implies that the diffusion of an intermediate into the cellular space is a rate limiting factor. Indeed, by applying the kinetics of elementary collisions, Pollard (1963) concluded that for cells larger than $1\mu\text{m}$ [c.f. Acinetobacter dimensions $1.5\text{-}2.5 \times 0.9\text{-}1.6\mu\text{m}$ (Baumann et al., 1968)], the diffusional term becomes a limiting step for the accomplishment of metabolic processes

and consequently a submicroscopic order is required.

By contrast, the concentrations of the glycolytic enzymes have been analysed in detail in the yeast Saccharomyces carlbergensis (Hess et al., 1969) and have been found to be in the range 10^{-5} - 10^{-4} M, thus the catalytic sites are equimolar with many glycolytic intermediates. Hess concluded from these data that the packing of the glycolytic enzymes in the cell is sufficiently dense to avoid any time delays by diffusion of intermediates.

e) Coordinate regulation

Regulation of an entire multienzyme complex could possibly be achieved by a single effector acting at a single site. Furthermore, if the formation of a complex were necessary either to produce an enzyme species with any activity at all or to elevate the efficiency of a pathway to a physiologically useful level, then the ligand-controlled association or dissociation of the members of a complex could provide an additional mechanism for the control of the pathway.

Using a partially purified arom complex from N.crassa, Welch & Gaertner (1976) presented evidence for coordinate activation of the five enzyme activities in the complex by DAHP (3-deoxy-D-arabino-heptulosonic acid 7-phosphate) which is the substrate of the first enzyme in the complex (Figure 2). However, Coggins & Boocock (1986) were unable to achieve such an effect of coordinate activation with a highly purified arom pentafunctional polypeptide and were critical of the methods used by Welch & Gaertner (1976).

Are enzyme complexes necessary for coordinate regulation? According to the theory of Kacser & Burns (1973, 1979) and Kacser (1983) all enzymes in a pathway contribute to flux control and changes in the activity of any component of that pathway merely redistributes the effects of the other enzymes in the pathway. This implies that the enzymes of a given pathway could be coordinately regulated even in the absence of complex

Figure 2 Aromatic amino acid biosynthesis and metabolism of
quininate and p-hydroxybenzoate

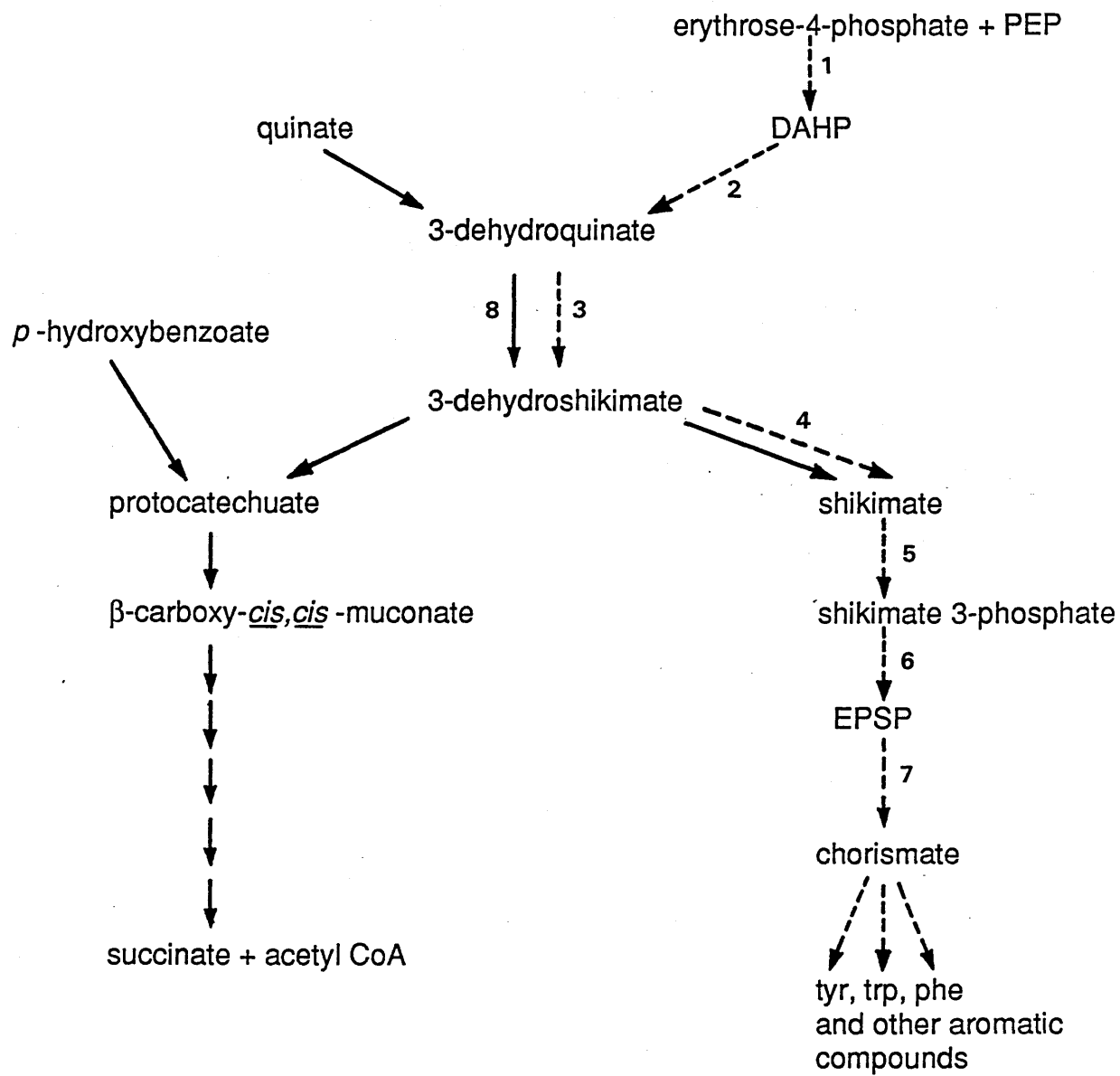
DAHP = 3-deoxy-D-arabino-heptulosonic acid 7-phosphate

EPSP = 5-enolpyruvoylshikimate 3-phosphate

- 1, DAHP synthase ; 2, 3-dehydroquininate synthase;
3, 3-dehydroquinase ; 4, shikimate dehydrogenase;
5, shikimate kinase ; 6, EPSP synthase;
7, chorismate synthase ; 8, catabolic 3-dehydroquinase.

Enzymes 2 - 6 are present on the multifunctional arom polypeptide.

- biosynthetic pathway
—→ catabolic hydroaromatic pathway



formation. Alternatively, if the activity of the 'rate limiting' enzyme was regulated then the enzyme pathway would be regulated.

1.5.3 Enzyme complexes - no advantage?

There is always the possibility that even although enzyme associations occur they may not confer any particular advantage on the cell. Enzyme associations could be evolutionary relics or a consequence of the process of enzyme synthesis. Another possibility is that enzyme interactions could be an inherent, but accidental, property of particular proteins and it could be that since enzyme interactions take place, the cell has found a use for them (Gould & Lewontin 1979).

1.5.4 Examples of 'soluble' enzyme interactions in eukaryotes

Evidence for the existence of weak enzyme interactions has been difficult to find. This may be due to the lability of the putative enzyme clusters but it may also reflect the transient nature of their association or, of course, the fact that they do not exist. It should be kept in mind that attempts to isolate transient species e.g. enzyme-substrate complexes have shown that this is not an easy task, yet such complexes obviously exist (Hess & Boiteux, 1972).

The situation in eukaryotic cells is complicated by the existence of a microtubular lattice (MTL) which is an elaborate network of protein strands that connect with almost all cytoplasmic ultrastructure (Wolosewik & Porter, 1979; Porter & Tucker, 1981). It is argued that classical 'soluble' enzymes are not freely diffusible because they are integral components of the MTL (Clegg, 1981, 1984a,b; Masters, 1981).

Several investigators (e.g. Knull et al., 1974; Masters, 1981) have suggested that the subcellular distribution of some enzymes may not be invariant but it may be a dynamic characteristic which alters in response to the changing requirements of the cell. The term 'ambiquitous' has been coined to describe the situation where the

distribution of an enzyme between soluble and particulate forms may vary with the metabolic status of the cell (Wilson, 1981).

1.5.4.1 Glycolytic enzymes in muscle

Most glycolytic enzymes appear to be associated with actin filaments in skeletal muscle (Clarke & Masters, 1975; Walsh et al., 1981). The extent of enzyme binding and the catalytic properties of the enzymes have been demonstrated to be strongly influenced by their substrates, other proteins, pH, ATP, Ca^{++} , Mg^{++} and electrical stimulation suggesting that enzyme function in vivo may be regulated by their interactions with the structural proteins (Knull et al., 1980; Bronstein & Knull, 1981).

1.5.4.2 Glycolytic enzymes in red blood cells

A particle containing glycolytic enzyme activity has been isolated from broken cell ghosts (Fossel & Solomon, 1978). The glycolytic complex consisting of glyceraldehyde-3-phosphate dehydrogenase, monophosphoglycerate mutase and phosphoglycerate kinase was shown to interact with the inner membrane and the interaction of the complex with the membrane was responsive to the application of ouabain to the outer membrane surface.

1.5.4.3 Glycolytic enzymes in the yeast *S.carlsbergensis*

Ultracentrifugation studies and kinetic studies on extracts of *S.carlsbergensis* were carried out in an attempt to show interactions between the glycolytic enzymes (Hess & Boiteux, 1972). However, even with the high enzyme concentrations used (140mg ml^{-1}), no aggregation of the enzymes was observed.

1.5.4.4 Trypanosome glycosome

The parasite *Trypanosoma brucei* consumes glucose at a rate 50 times that of most mammalian cells and this may be possibly due to the compartmentation of the glycolytic enzymes in an organelle called the glycosome (Opperdoes et al., 1977a,b; Opperdoes & Borst, 1977; Oduro et al., 1980). Pulse labelling experiments showed that the glycolytic

carbon flow in T.brucei goes through a compartment which is separated from the rest of the cell and involves approximately 20-30% of the total glycolytic intermediates (Visser et al., 1981). Crosslinking studies confirmed the close proximities of the glycolytic enzymes in the glycosome (Aman et al., 1985), yet Aman & Wang (1986) were unable to demonstrate any substrate channeling in a crosslinked complex. After it had been reported that even when the glycosomal membrane had been destroyed all the enzymes in the complex remain tightly bound together (Musset & Oppendoes, 1983), Ottaway (1985) raised the question that if this is so then why is there a need for such a membrane?

1.5.4.5 Krebs cycle enzymes in rat liver mitochondria

Srere (1972) first proposed that the 'soluble' Krebs cycle enzymes exist as a multienzyme complex near, or on, the inner (matrix) side of the inner membrane of rat liver mitochondria. After being unable to demonstrate any high molecular mass aggregate of the Krebs cycle enzymes by studying mitochondrial extracts on sucrose density gradient centrifugation and gel filtration chromatography (Srere et al., 1978), Srere and others have subsequently used a variety of techniques to demonstrate a Krebs cycle enzyme complex (Srere, 1985).

a) Swollen mitochondria

In swollen mitochondria, a drastic reduction in the rate of oxidation of a substrate would be expected since the mean free path between an intermediate and the next enzyme would be increased and the proposed Krebs cycle enzyme complex would be dissociated. Matlib & Srere (1976) demonstrated that by swelling mitochondria, at least a three fold increase in the mitochondrial volume was achieved and this resulted in a significant reduction in the rate of oxidation of substrates.

b) Crosslinking

A variety of chemical crosslinkers were used in intact mitochondria to study matrix proteins (D'Souza & Srere, 1983). It was anticipated that if the Krebs cycle enzymes interact and are localised near the

surface of the inner membrane, chemical crosslinkers would stabilise such interactions. Indeed, disorganisation of matrix proteins, by either swelling mitochondria or by sonication, reduced the degree of crosslinking and it was concluded that the Krebs cycle enzymes are preferentially localised within the matrix of rat liver mitochondria next to the inner membrane (D'Souza & Srere, 1983).

c) Studies with purified enzymes

Many workers have used purified enzymes to study enzyme interactions (Srere, 1985) e.g. specific interactions have been demonstrated between fumarase and malate dehydrogenase (Beekmans & Kanarek, 1981), citrate synthase and malate dehydrogenase (Halper & Srere, 1977), pyruvate dehydrogenase complex and citrate synthase (Sumegi *et al.*, 1980) and succinate thiokinase and α -ketoglutarate dehydrogenase complex (Porpaczy *et al.*, 1983). Several techniques were used in these studies including chemical crosslinking of purified enzymes, enzyme interactions in polyethylene glycol and immobilised enzymes (see (d) and (e) below).

d) Use of polyethylene glycol

The presence of polyethylene glycol (PEG) in dilute solutions of globular proteins results in the proteins being sterically excluded from regions of the aqueous solvent occupied by the synthetic polymer thus proteins are confined to a reduced volume (Middaugh *et al.*, 1979; Knoll & Hermans, 1983). By exploiting this property of PEG, Halper & Srere (1977) were able to demonstrate a specific interaction between purified citrate synthase and malate dehydrogenase.

e) Immobilised enzyme systems

An immobilised enzyme system of malate dehydrogenase and citrate synthase (either as co-immobilised separate enzymes or as aggregates immobilised to a particular support such as Sepharose beads) was shown to operate at a faster rate than the equivalent amounts of free enzymes (Koch-Schmidt *et al.*, 1977).

Being aware that many of the enzyme interactions illustrated here (1.5.4.5 c - e) were not observed at physiological ionic strength, Srere (1980, 1981, 1982) argued firstly that we do not have an accurate assessment of ionic strength within the cell; and secondly, since cellular protein concentrations are quite high (20-50%) (Sols & Marco, 1970; Ottaway & Mowbray, 1977; Srere, 1980, 1982, 1984, 1985) and since molecular crowding (Minton, 1981) can markedly affect weak protein interactions, these interactions are likely even at physiological ionic strength; and finally, the interactions are specific for enzymes of sequential metabolic reactions.

1.5.5 Examples of 'soluble' enzyme interactions in bacteria

Since bacteria lack compartmentation by subcellular organelles, enzyme complex formation could conceivably be an important method of metabolic compartmentation.

1.5.5.1 Aromatic amino acid biosynthesis and metabolism of quinate

The biosynthesis of the aromatic amino acids phenylalanine, tyrosine and tryptophan has a common stretch of reaction sequence consisting of seven enzyme steps (Figure 2). This pathway occurs in microorganisms and higher plants but is absent from animals. In fungi e.g. N.crassa, five of these enzyme activities (enzymes 2-6 in Figure 2) are present on a single polypeptide chain (Berlyn et al., 1970; Lumsden & Coggins, 1977). A bifunctional polypeptide with 3-dehydroquinase and shikimate dehydrogenase activities (enzymes 3 and 4 in Figure 2) has been found in plants (Berlyn et al., 1970; Polley, 1978). These enzymes (enzymes 2-6 in Figure 2) were found to be separate in E.coli and five other bacteria which were studied (Berlyn & Giles, 1969).

However, it has been suggested that in A.calcoaceticus the regulatory mechanism necessary for the protection of biosynthetic intermediates (particularly 3-dehydroquinase and 3-dehydroshikimate) in the presence of inducible enzymes catalysing their degradation (Figure 2)

may involve a multienzyme complex of the biosynthetic enzymes analogous to those found in fungi (Ingledew et al., 1971). Evidence for this was somewhat indirect and came from mutants which had lost the inducible catabolic dehydroquinase (enzyme 8, Figure 2), although normal levels of the biosynthetic enzyme were present (Ingledew et al., 1971). The mutants were completely unable not only to grow on quinate but also to produce enough protocatechuate to induce the other enzymes of the hydroaromatic pathway when growing on succinate in the presence of quinate. Further indirect evidence of 3-dehydroshikimate channeling came from Tresguerres et al. (1972) who showed differential utilisation of exogenous and dehydroquinase-derived dehydroshikimate. Berlyn & Giles (1973), however, later reported that under conditions of sucrose density gradient centrifugation where the biosynthetic enzyme complex was readily demonstrable in N.crassa, the A.calcoaceticus enzymes were separable, but they admitted that physical compartmentation of the components of the two pathways could predominate but would probably be destroyed after disruption of the bacteria.

1.5.5.2 Enzymes of the β -ketoadipate pathway in P.putida

Metabolic compartmentation of β -carboxy-cis,cis-muconate (Figure 2) has been suggested (Meagher et al., 1972) on the basis that exogenously supplied β -carboxy-cis,cis-muconate induced the synthesis of an uptake system that was not induced by β -carboxy-cis,cis-muconate when it was supplied endogenously by the catabolism of p-hydroxybenzoate (Figure 2). This indirect evidence was somewhat substantiated when copurification of four enzymes of the β -ketoadipate pathway (metabolism of protocatechuate to succinate and acetyl CoA, Figure 2) was observed on a DEAE cellulose column (Meagher et al., 1972). After exposure to ammonium sulphate, the four enzymes eluted from the column as sharply defined zones of activity suggesting that a non-covalent enzyme aggregate exists but is destroyed by ammonium sulphate (Meagher et al., 1972).

1.5.5.3 Organisation of the glycolytic enzymes in E.coli

Compartmentation of glycolytic metabolism in E.coli has been suggested on the basis of metabolic experiments using competing labelled and unlabelled substrates (McBrien & Moses, 1968; MacNab et al., 1973). In the first of these experiments (McBrien & Moses, 1968), resting cultures, treated with chloramphenicol to prevent additional enzyme synthesis, were divided into several identical subcultures all supplied with the same mixture of glucose, galactose and lactose and each culture also received one of those sugars labelled with ^{14}C . The cultures were found to exhibit differences both in their preference for labelled hexose and in their metabolic product labelling patterns for the Krebs cycle intermediates ^{14}C -citrate and ^{14}C -succinate and the amino acid ^{14}C -glutamate (McBrien & Moses, 1968). In a later series of experiments (MacNab et al., 1973) the incorporation of ^{14}C into protein amino acids was measured using cultures of growing bacteria. The purpose of these experiments was to investigate the possibility of the preferential metabolism of intermediates derived from maltose carbon (entering glycolysis at the level of hexose) compared with the glycerol carbon (joining glycolysis at the level of triose phosphate isomerase). Although maltose and glycerol were shown to be equivalent carbon sources, since they supported the same growth rate and the same amount of carbon was required for growth in each case, more maltose carbon was incorporated into amino acids than glycerol carbon. These results indicated a substantial degree of channeling.

In an attempt to find more concrete evidence for the existence of a glycolytic enzyme complex, Mowbray & Moses (1976) undertook a study of these enzymes involving gentle disruption of the bacteria using lysozyme, followed by differential centrifugation and gel filtration chromatography of the extracts so formed. After differential centrifugation, 14-20% of the glycolytic enzyme activities were recovered in the high speed

pellet although most of this activity dissociated on washing the pellet. Evidence of a high M_r aggregate of the glycolytic enzymes was obtained on gel filtration chromatography. However, in neither of these studies was any comparison with control enzymes (enzymes from unrelated parts of metabolism) included and therefore the possibility that these results are due to non-specific binding cannot be excluded.

Gorringe & Moses (1980) claimed to have reconstituted a high M_r particle, containing glycolytic enzyme activity, from a concentrated sample of the individual enzymes from a lysed spheroplast preparation. Throughput activity of the glycolytic pathway was demonstrated in this high M_r species although no throughput activity was observed for a sample of individual glycolytic enzymes at the same protein concentration. It was concluded from this that for throughput activity to be observed, not only had all the glycolytic enzymes to be present in sufficient quantity but they also had to be structurally integrated into a specific association.

However, Kingdon (1984) was unable to repeat the results obtained on gel filtration chromatography reported by Mowbray & Moses (1976) and Gorringe & Moses (1980) and concluded that the enzyme complexes apparently observed were due to enzyme-cellular debris association and whole cell contamination of the experimental samples. Kingdon (1984) was particularly critical of the throughput experiments on the glycolytic particle reported by Gorringe & Moses (1980); radiolabelled glucose was used in these studies, however most glucose appears intracellularly as glucose-6-phosphate having been transported across the bacterial membrane by the phosphotransferase system (Waywood & Sleeves, 1980; Begley *et al.*, 1982). The formation of pyruvate in these throughput experiments was monitored by the addition of aminotransferase and quantifying the amount of ^{14}C -alanine formed by paper chromatography (Gorringe & Moses, 1980).

However, Kingdon (1984) discovered that the ^{14}C -labelled product observed on the paper chromatograms was in fact ^{14}C -lactate which had been produced by viable bacteria which contaminated the sample.

Nevertheless, in experiments involving sucrose density gradient centrifugation studies following in vivo protein crosslinking with dimethylsuberimide (DMSI), there was evidence which suggested that a soluble enzyme-enzyme association occurs between triose phosphate isomerase, glyceraldehyde 3-phosphate dehydrogenase and a third unknown component with an apparent M_r of 150,000 (Kingdon, 1984). It was suggested that this complex might serve to reduce the intracellular concentration of the toxic glycolytic intermediate, glyceraldehyde 3-phosphate (Kingdon, 1984).

1.5.5.4 Association of the Krebs cycle enzymes in E.coli

Using the methodology of Mowbray & Moses (1976) and Gorringe & Moses (1980), Barnes & Weitzman (1986) and Weitzman & Barnes (1985) reported evidence for a high M_r aggregate of the Krebs cycle enzymes in E.coli. The high M_r species was isolated from lysed spheroplasts by gel filtration chromatography in the presence of 20% (v/v) glycerol. Addition of 14% (w/v) PEG or BSA (10mg ml^{-1}) produced a 2-3 fold increase in the yield of the high M_r species from gel filtration chromatography; however, no high M_r species were detected in the absence of 20% (w/v) glycerol or in the presence of 1M KCl and the yield of the high M_r species was reduced by 50% when the pH was changed from 7.5 to 6.5 or 8.9 (Barnes & Weitzman, 1986). There was no evidence of a Krebs cycle enzyme aggregate when the bacteria were disrupted by sonication or in the French pressure cell. As with the experiments on the glycolytic enzymes (Mowbray & Moses, 1976; Gorringe & Moses, 1980) the behaviour of control enzymes (from unrelated parts of metabolism) was not reported. However, Barnes & Weitzman (1986) did add lactate dehydrogenase (from rabbit muscle) to the bacterial lysate to check for non-specific binding and reported that this enzyme

did not elute at a position corresponding to a high M_r . Similar results indicative of a Krebs cycle enzyme aggregate were said to be obtained with A.calcoaceticus, P.aeruginosa and B.subtilis (Weitzman & Barnes, 1985; Barnes & Weitzman, 1986). Since Barnes & Weitzman (1986) followed the protocols outlined by Mowbray & Moses (1976) and Gorringer & Moses (1980), there is the possibility that these results are due to whole cell contamination of the experimental samples.

1.5.5.5 Association of the enzymes of the mandelate pathway in P.putida

Halpin et al. (1981) have reported evidence for the physical association of the enzymes of the mandelate pathway in P.putida on the basis of experiments involving the crosslinking reagent dimethylsuberimide (DMSI). The strategy behind using crosslinking reagents in these studies is that if enzyme interactions exist it is hoped that the crosslinker would provide sufficient stabilisation to allow their isolation and characterisation.

When extracts from control and DMSI-treated bacteria were subjected to differential centrifugation, there was a 9-25 fold increase in the soluble enzyme activities recovered in the pellet fraction (where most of the LMDH activity was present) after DMSI treatment. In addition, when extracts from bacteria which had been treated with DMSI in vivo were subjected to gel filtration chromatography, an extra peak of enzyme activities appeared in the void volume which in control extracts had only LMDH activity. This was taken to indicate the presence of a high M_r aggregate of the mandelate enzymes although, again, these workers did not pay much attention to the need to follow the distribution of control enzyme in these gel filtration experiments (Halpin et al., 1981).

The crosslinked complex of the mandelate enzymes was also studied by ^{13}C -NMR spectroscopy (Halpin et al., 1981). On oxygenation of the isolated complex, DL- $[\alpha\text{-}^{13}\text{C}]$ -mandelate was converted to $[\alpha\text{-}^{13}\text{C}]$ -benzoate; $[\alpha\text{-}^{13}\text{C}]$ -phenylglyoxylate and $[\alpha\text{-}^{13}\text{C}]$ -benzaldehyde were also detected.

This confirmed that all the enzymes necessary for the metabolism of mandelate to benzoate were present in the high M_r material. However, it was also reported that in at least one experiment, $[^{13}\text{C}]$ -bicarbonate was detected after oxygenation of the isolated complex suggesting that the enzymes of the 'catechol pathway' (Figure 1) were also present in the high M_r material. There is also the possibility that the mandelate enzymes as well as other unrelated 'soluble' enzymes had been randomly crosslinked since glutamine synthetase activity was detected in the isolated high M_r material, although Halpin *et al.* (1981) did not place any emphasis on this observation. The results presented by Halpin *et al.* (1981) are far from conclusive and it was not possible to determine whether the mandelate enzymes were complexed to each other or to other cellular components.

1.6 Aims

The primary aim of this study was to determine whether or not the enzymes of the mandelate pathway in A.calcoaceticus exist as a multi-enzyme complex. If there was evidence for such a complex then the next objective would have been to isolate it and characterise it in terms of size, composition and kinetic properties; however, as it turned out, there was no sound evidence for the existence of a complex.

It was hoped that techniques such as gel filtration chromatography, density gradient centrifugation and ultracentrifugation would be useful for testing whether there was evidence for co-purification of the mandelate enzymes. For these co-purification studies it was thought that a gentle method of disruption would be necessary and so the first work that was undertaken was to develop a method for disrupting A.calcoaceticus after lysozyme treatment.

It was realised that it would be necessary to develop appropriate methods for assaying a range of enzymes and for crosslinking experiments either in vivo or using bacterial extracts.

A subsidiary aim was to purify LMDH with a view to raising antibodies against the enzyme which could be useful in identifying crosslinked species or in immunoprecipitation studies on the native complex. An additional reason for purifying LMDH was so that it could be characterised thus making it possible to compare further the stereospecific mandelate and lactate dehydrogenases.

2.1 Materials

All reagents were the best grade available commercially. With the exceptions of the materials listed below, reagents were obtained from BDH Chemicals Ltd., Poole, Dorset, BH12 4NN.

Phenylene dimaleimide - Aldrich Chemical Co.Ltd., The Old Brickyard, New Road, Gillingham, Dorset SP8 4JL.

Bovine serum albumin (Fraction V) - Armour Pharmaceuticals Co.Ltd., Eastbourne, Sussex BN22 9AG.

HRP colour development reagent - Bio-Rad Laboratories Ltd., Watford, Hertfordshire.

Aldolase; lactate dehydrogenase; pyruvate kinase (all from rabbit muscle) - Boehringer Corp., Ltd., Lewes, East Sussex BN7 1LG.

Freund's complete and incomplete adjuvant - Difco Laboratories, Detroit, Michigan 48201, U.S.A.

D-Mandelic acid; L-mandelic acid; phenylglyoxylic acid - Fluorochem Ltd. (Fluka), Glossop, Derby SK13 9NU.

Ultrogel Aca34 and HA Ultrogel - LKB Instruments Ltd., 232 Addington Road, South Croydon, Surrey.

2-Phenylpropionic acid - K + K, Kodak Ltd., Kirby, Lancs L33 7UK.

Nutrient broth (CM1); nutrient agar (CM3); agar No.1 - Oxoid Ltd., Basingstoke, Hants RC24 OPW.

DEAE Sephacel; gel filtration calibration proteins; SDS PAGE calibration proteins; Percoll; density gradient marker beads; Polybuffer 74 - Pharmacia (Great Britain) Ltd., Prince Regent Road, Hounslow, Middlesex.

L-Glutamic acid.HCl - T.J. Sas and Son Ltd., London WC1.

HRP donkey anti-rabbit IgG; normal rabbit serum; normal donkey serum - Scottish Antibody Production Unit, Law Hospital, Carlisle.

Aldehyde dehydrogenase (yeast); alcohol dehydrogenase (horse liver);

Coomassie Brilliant Blue G250; deoxyribonuclease; 3-deoxy-2-keto-octonic acid; D,L-isocitric acid; lysozyme; Lubrol PX; NAD; NADP; NADH; PMS;

phosphoenol pyruvate potassium salt; protein A-Sepharose; ribonuclease; SDS PAGE calibration proteins; TPP (cocarboxylase); Triton X-100; Tween 20 - Sigma Chemical Co.Ltd., Poole, Dorset BH17 7NH.

Dimethylmalonimide; dimethylsuccinimide; dimethylglutarimide; dimethyladipimide; dimethylpimelimide; dimethylsuberimide - gifts from Dr. J.R. Coggins, Department of Biochemistry, University of Glasgow.

Anti-DMDH; anti-PGDC - gifts from Dr. I.D. Hamilton and Dr. M.M. Barrowman, Department of Biochemistry, University of Glasgow.

2.2 Microbiological techniques

2.2.1 Microorganisms

The bacteria used in this work are listed in Table 1. All strains except mutant strains C1005, C1123 and C1219 were maintained in 10ml Oxoid nutrient broth (CM1), stored at 4°C. Mutant strains C1005, C1123 and C1219 were maintained in 10ml complex medium plus D,L-mandelate, instead of nutrient broth in order to maintain the selection pressure during storage and so avoid the outgrowth of non-constitutive revertants. Subcultures were made into Oxoid nutrient broth (CM1) or complex medium plus D,L-mandelate at 6-12 monthly intervals. In addition, constitutive strains were periodically replated on salts medium containing 2-phenylpropionate and phenylglyoxylate. Colonies were then picked off and cultured in complex medium plus D,L-mandelate and stored at 4°C.

2.2.2 Characterisation of bacteria

Characteristics of the various strains were checked with the aid of Oxi-Ferm and Entero diagnostic tubes (Roche Products Ltd., PO Box 8, Welwyn Garden City, Herts AL7 3A7). Oxidase reactions (Cowan & Steel, 1965) and the appearance of colonies on nutrient agar were also examined.

2.3 Sterilisation

2.3.1 Moist heat

Heat-stable solutions were autoclaved. Media containing carbon sources were autoclaved at 109°C (except for complex media) and all other

Table 1 Strains of bacteria1. Acinetobacter calcoaceticus

- NCIB 8250 Wild type strain, inducible for LMDH, PGDC and BDH I
 (Fewson, 1967b; Livingstone et al., 1972)
- NCIB 8250 Mutant strain C1005 (previously HMM5) (NCIB 11339)
 constitutive for LMDH, PGDC and BDH I (Fewson et al., 1978)
- NCIB 8250 Mutant strain C1219 (NCIB 11457), constitutive for LMDH,
 DMDH, PGDC and BDH I (Hills & Fewson, 1983a)
- NCIB 8250 Mutant strain C1123, constitutive for DMDH, PGDC, BDH I,
 no LMDH activity present (Hills & Fewson, 1983a)
- NCIB 8250 Mutant strain C1408, constitutive for PGDC and BDH I,
 no LMDH or DMDH activities present (Hills & Fewson, 1983a)

2. Pseudomonas putida

- NCIB 9494 Wild type strain (previously A3.12), inducible for the
 mandelate enzymes (Hegeman, 1966a)

solutions at 109°C or 120°C for times established by Fewson (unpublished results). Efficiency of sterilisation was verified by using Browne's tubes (Type three, A. Browne Ltd., Chancery Street, Leicester).

2.3.2 Dry heat

Pipettes were wrapped in either Kraft paper or placed in metal cannisters before sterilisation. Each sterilisation (160°C, 1.75h) was checked by including a Browne's tube (Type one).

2.3.3 Filtration

Volatile and heat-labile compounds were sterilised by filtration. Large volumes of media were sterilised through Millipore filters (GSWPO 4700, 0.2µm pore size; Millipore Ltd., Abbey Road, London). For volumes of 100ml or less, Nalgene disposable sterile filter units (0.2µm pore size; Sybron Corp., Rochester, N.Y., U.S.A.) were used.

2.3.4 Ethylene oxide

Plastic pipettes and other 'disposable' apparatus were sterilised with ethylene oxide. All items were sealed with polythene film and exposed to ethylene oxide (Anprolene) for 12h in a sterilising box (AN74; H.W. Anderson Products Ltd., Clacton-on-Sea, Essex). Sterilisation was verified by an Anprolene exposure indicator (AN85) or by a steritest unit (AN80). All apparatus was aired for at least 24h prior to use to remove residual gas.

2.4 Media

2.4.1 Minimal medium containing mandelate

This medium was adapted from that used by Hegeman (1966a) and contained (gl⁻¹) D,L-mandelate 1.52 (10mM); (NH₄)₂SO₄, 1; KH₂PO₄, 3.4; Na₂HPO₄, 3.55; and 2ml Chel metals l⁻¹. Chel metals was prepared as follows.

Nitrilotriacetic acid (Chel) (80g) was dissolved in 1M NaOH (625ml) and the solution was adjusted to pH7.0 with 5M HCl. Salts were then added in the following order:- FeSO₄·7H₂O, 1.1g; Na₂MoO₄·2H₂O, 50mg; MnSO₄·4H₂O, 50mg; ZnSO₄·7H₂O, 50mg; CuSO₄·5H₂O, 25mg; CoCl₂·6H₂O, 25mg; and the solution was made up to 1l with distilled water and autoclaved (Beggs & Fewson, 1977).

The minimal medium containing mandelate was adjusted to pH6.9 with NaOH, made to the correct final volume with distilled water and autoclaved. Immediately before inoculation, sterile 2% (w/v) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (20ml l^{-1}) was added.

2.4.2 Basal medium

The basal medium contained 2g of KH_2PO_4 and 1g of $(\text{NH}_4)_2\text{SO}_4$ per litre, adjusted to pH7.0 with NaOH.

2.4.3 Salts medium

The salts medium contained 2g of KH_2PO_4 and 1g of $(\text{NH}_4)_2\text{SO}_4$ per litre, adjusted to pH7.0 with NaOH, and 20ml 2% (w/v) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ per litre which was added after autoclaving, thus 5mM L-glutamate/salts medium refers to 5mmol L-glutamate per litre of salts medium (Kennedy & Fewson, 1968a).

2.4.4 Nutrient broth

Nutrient broth (Oxoid CM1; 13g l^{-1}) was dispensed in 10ml volumes in universal bottles for storage of cultures.

2.4.5 Complex medium

The complex medium (Allison *et al.*, 1985a) contained (g l^{-1}): Oxoid No.1 nutrient broth, 26; KH_2PO_4 , 2; $(\text{NH}_4)_2\text{SO}_4$, 1; L-glutamic acid. HCl, 0.9; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.4. This medium was supplemented with D,L-lactate (1.5g l^{-1}) and/or D,L-mandelate (1.5g l^{-1}) or phenylglyoxylate (0.75g l^{-1}) as required, adjusted to pH7.0 with NaOH, made to the correct final volume with distilled water and autoclaved.

2.5 Agar plates

2.5.1 Nutrient agar plates

The agar (Oxoid CM3; 28g l^{-1}) was boiled and stirred to dissolve it and then autoclaved. The sterile molten medium was cooled to 55°C and poured into petri dishes (9cm diameter - Sterilin Ltd., Teddington, Middlesex). Plates were left inverted for 24h at 30°C to dry.

2.5.2 Agar plates containing defined media

Oxoid agar No.1 [L11; 1.5% (w/v)] was mixed with KH_2PO_4 (2.0g l^{-1}) and $(\text{NH}_4)_2\text{SO}_4$ (1.0g l^{-1}) in addition to the appropriate carbon source which had been adjusted to pH7.0 with NaOH and then autoclaved. If the carbon source was heat-labile it was sterilised by filtration and added to the sterile molten agar mixture at 55°C . Before pouring into the plates, 20ml 2% (w/v) sterile $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ was added to each litre of medium. The plates were then dried at 30°C for 24h.

2.6 Optical density measurements

Optical densities (O.D.) of bacterial suspensions were estimated by measuring the OD_{500} in a Pye Unicam SP30 Spectrophotometer (Pye Unicam Instruments Ltd., 576 West Harbour Road, Granton, Edinburgh EH5 1PP). Dense suspensions were diluted with the appropriate medium so that the OD_{500} was always less than 0.6 when read in the spectrophotometer.

2.7 Growth of bacteria

2.7.1 General growth conditions

Culture volumes of 50ml or 500ml were grown in 250ml or 2 litre conical flasks, respectively, stoppered with polystyrene foam bungs (A.& J. Beveridge Ltd., 5 Bonnington Road Lane, Edinburgh EH6 5BP). The cultures were usually grown on a rotary shaker (M.K.V. Orbital Shaker, L.H. Engineering Co.Ltd., Stoke Poges) moving at about 120 rpm in rooms maintained at 23°C or 30°C as appropriate.

Cultures of 4 litre volumes were grown in 10 litre flat-bottomed flasks each having a plug of non-adsorbent cotton wool through which passed a plugged glass pipette (10ml). The cultures were grown at 30°C under conditions of vigorous aeration produced by a 45mm polypropylene-coated magnetic bar stirring at approximately 300rpm on the apparatus described by Harvey et al. (1968). Air (400ml min^{-1}) was passed through the glass pipette over the culture.

Larger amounts of bacteria were grown in a 10 litre fermenter (Braun Biostat V., F.T. Scientific Instruments, Tewkesbury, Glos., UK) which contained 10 litres of complex medium and 50ml of sterile 1% (v/v) poly (propylene glycol) 2025 as antifoam. A 20 litre reservoir containing the same medium was connected to the fermenter. The fermenter was operated at 23°C or 30°C as appropriate with an aeration rate of 4 litres of sterile air min⁻¹ and stirring at setting 2.5 (approx. 350 rpm). After 16h growth, when the apparent OD of the culture at 500nm was at least 2.0, 9 litres of the culture was harvested and 9 litres of fresh medium was added to the fermenter from the reservoir. After another period of growth, this process was repeated.

2.7.2 Growth of *A.calcoaceticus*, mutant strain C1005 on complex medium

Inocula were prepared by aseptically transferring 0.1ml of a stock culture into 50ml of medium (in 250ml flasks) and growing at 23°C with shaking for 48h or at 30°C with shaking for 24h. These cultures were used as inocula (2.5%, v/v) for 400ml of medium (in 2 litre conical flasks) which were then incubated on an orbital shaker at 120 rpm for 24h at 23°C or 16h at 30°C. These cultures were then either harvested or used to inoculate the fermenter containing 10l of complex medium.

2.7.3 Growth of wild type *A.calcoaceticus* NCIB 8250 on 5mM succinate/salts medium

A stock nutrient broth culture was inoculated (0.2%, v/v) into 50ml succinate/salts medium (in a 250ml conical flask) and grown for 24h at 30°C. All of this culture was then inoculated into 4 litres of 5mM succinate/salts medium in a 10 litre flat-bottomed flask, plugged with non-adsorbant cotton wool and grown under conditions of vigorous aeration for 16h at 30°C.

2.7.4 Growth of *P.putida* NCIB 9494 on minimal mandelate medium

A stock nutrient broth culture was inoculated (0.2%, v/v) into 50ml of minimal medium containing mandelate and grown for 24h at 30°C with shaking. This culture was used as the inocula (2.5%, v/v) for 400ml of medium (in 2 litre flasks) which were incubated at 30°C with shaking. These cultures were harvested when the OD₅₀₀ of the culture reached 0.8 (after about 5h).

2.8 Harvesting and storage of bacteria

Cultures were harvested by centrifugation in 750ml polypropylene bottles (maximum volume 500ml; 69650, MSE Ltd., Buckingham Gate, London) at 5200rpm for 20 min at 4°C in an MSE Mistral 6L centrifuge. Each pellet was resuspended in ice-cold, sterile basal medium and recentrifuged in a 50ml polypropylene centrifuge tube (59407, MSE) at 12,000g for 30 min at 4°C in an MSE 18 centrifuge. The supernatant was decanted and the washed bacterial pellet was either used immediately or stored at -20°C until required.

2.9 Disruption of bacteria

2.9.1 Ultrasonic disruption

Bacteria were resuspended in an appropriate buffer (usually 0.05M potassium phosphate buffer, pH7.5) to a final concentration of 0.25g wet weight ml⁻¹. Five-seven ml were placed in a 10ml vial and positioned in a chilled brass holder (Holms & Bennett, 1971). This was screwed onto the horn of a 13mm probe of the Dawe Soniprope (Type 7532A; Dawe Instruments Ltd., London) and lowered into an ice-water slurry. *A.calcoaceticus* was disrupted at a current of 80W for 5 x 30s periods alternated with cooling periods of 30s. *P.putida* was also disrupted at a current of 80W but for 3 x 30s periods alternated with cooling periods of 30s. The homogenate was centrifuged at 15,000g for 20 min at 4°C in an MSE Highspeed 18 centrifuge to remove intact bacteria and debris. The supernatant was stored at -20°C.

2.9.2 French press disruption

Bacteria were resuspended in an appropriate buffer to a final concentration of $0.5\text{g wet weight ml}^{-1}$ and disrupted by two or four passages through the French Press (Cat. No.4-3398A, Aminco, Silver Spring, Maryland, USA) at a pressure of 98MPa ($14,300\text{ lb in}^{-2}$). The homogenate was centrifuged at $15,000\text{g}$ for 20 min at 4°C in an MSE 18 Highspeed centrifuge to remove intact bacteria and debris.

2.9.3 Disruption by lysozyme treatment

2.9.3.1 This method was slightly modified from that of Scott *et al.* (1976). Freshly harvested bacteria were washed three times in 0.05M Tris/HCl buffer, pH7.8 and resuspended ($50\text{mg wet wt ml}^{-1}$) in about 200ml of 0.05M Tris/HCl buffer, pH7.8 containing 0.6M sucrose. The suspension was incubated with lysozyme (10mg/g wet wt) for 150 min at 23°C before lysis of the spheroplasts by the addition of 10 volumes of cold distilled water. Deoxyribonuclease ($2\text{mg/g original wet wt}$) was added to decrease the viscosity. After the mixture had been stirred for 90 min at 4°C , intact bacteria were removed by centrifugation at $15,000\text{g}$ for 20 min at 4°C in an MSE 18 Highspeed centrifuge.

2.9.3.2 Alternatively, after incubating the bacterial suspension with lysozyme for 150 min at 23°C (Methods 2.9.3.1), the spheroplasts were harvested by centrifuging at $15,000\text{g}$ for 20 min at 4°C in an MSE 18 Highspeed centrifuge. The spheroplasts were washed once by resuspension/centrifugation in 0.05M Tris/HCl buffer, pH7.8 containing 0.6M sucrose. The spheroplast sludge was homogenised for 45s at top speed on a Potter Elvehjem homogeniser, then a crystal of deoxyribonuclease was added and the homogenate was incubated at 23°C for 5 min. The homogenising and deoxyribonuclease treatment was repeated twice (Mowbray & Moses, 1976). Finally, intact bacteria were removed by centrifuging at $15,000\text{g}$ for 20 min at 4°C in an MSE 18 Highspeed centrifuge.

2.10 Separation of outer and inner membranes

This method was adapted from that described by Scott et al. (1976). Freshly harvested bacteria (10g) were disrupted by lysozyme treatment (Methods 2.9.3.1). Membranes were collected from the resulting low speed supernatant by centrifuging at $65,000g_{av}$ (25K rpm, 10 x 100ml Prepsin rotor) for 120 min at 4°C and were resuspended in 0.05M potassium phosphate buffer, pH7.5 containing deoxyribonuclease and ribonuclease (0.1mg of each/g original wet wt). After the mixture had been stirred for 30 min at 4°C , the membranes were collected as before and washed twice by resuspension/centrifugation and were resuspended in a small volume (approx. 12ml) of 0.05M potassium phosphate buffer, pH7.5 containing $20\mu\text{g}$ of chloramphenicol ml^{-1} . Centrifugation of this crude membrane fraction at top-speed (approx. $15,000g$) in an Eppendorf 3200 centrifuge for 10 min resulted in the sedimentation of the cytoplasmic membranes and the outer membranes remained in the supernatant fraction. The cytoplasmic membranes were washed once by resuspension/centrifugation, and were resuspended in 0.05M potassium phosphate buffer, pH7.5 containing $20\mu\text{g}$ of chloramphenicol ml^{-1} . The outer membranes were recovered from the pooled supernatant fractions by centrifuging at $65,000g_{av}$ for 120 min and were resuspended in 0.05M potassium phosphate buffer, pH7.5 containing $20\mu\text{g}$ of chloramphenicol ml^{-1} .

2.11 Preparation of 'wall + membrane' fractions

2.11.1 The low speed supernatant resulting from bacterial disruption (Methods 2.9) was centrifuged at $105,000g_{av}$ (40K rpm, Ti50 rotor) for 60 min at 4°C and the pellet washed three times by resuspension and centrifugation in the appropriate buffer.

2.11.2 Alternatively, the low speed supernatant was centrifuged at $165,000g_{av}$ (40K rpm, Ti60 rotor or 50K rpm, Ti50 rotor) for 150 min at 4°C and the pellet washed twice by resuspension and centrifugation.

2.12 Toluene treatment of intact bacteria in the presence of polyethylene glycol (PEG)

Intact bacteria were treated with toluene according to the method described by Beggs & Fewson (1977) except that buffers contained 8.5% (w/v) PEG (Matlib *et al.*, 1977). Bacteria (3.8ml; 0.8g wet wt ml⁻¹) were pipetted onto 200µl 4% (v/v) toluene in ethanol. The mixture was vortexed for 30s and incubated at 27°C in a water bath for 90 min.

2.13 Electron microscopy

All electron microscopy was carried out by Mr. I. Montgomery, Physiology Department, University of Glasgow. Membrane fractions were negatively stained with 1.5% phosphotungstic acid (pH7.0). After incubating for 1 min at room temperature, excess fluid was removed and the grids were air dried.

2.14 Crosslinking with dimethylimidates

Crosslinking was carried out by the method developed by Dr.J.R. Coggins (Personal communication and Coggins, 1978).

2.14.1 Stock solutions

(a) TNM buffer, pH8.0 (0.05M Triethanolamine.HCl buffer, pH8.0 containing 0.1M NaCl and 0.01M MgCl₂).

(b) 0.4M NaOH.

(c) 2 x TNM buffer (0.1M Triethanolamine.HCl buffer, pH8.0 containing 0.2M NaCl and 0.02M MgCl₂).

(d) Dissolving mix: this was prepared by mixing 1 volume of 0.4M NaOH and 1 volume of 2 x TNM buffer.

(e) 0.1M stock dimethylimide solution: the solid dimethylimidates were stored at -20°C and since these reagents are very hygroscopic, the stock bottles were allowed to reach room temperature for approx 1h and the bottles were not opened for longer than necessary. A small amount of the dimethylimide was transferred to a stoppered bottle, weighed accurately and an appropriate volume of dissolving mix (d) was added to give a 0.1M

solution. Prepared this way, the pH of the stock solution was pH8.0. The dimethylimidate solution was used immediately or within 5 min of preparation.

2.14.2 Crosslinking aldolase with DMSI

A sufficient volume of 0.1M DMSI (Methods 2.14.1) was added to a solution of aldolase ($1-2\text{mg ml}^{-1}$) in TNM buffer, pH8.0, to give a final DMSI concentration of 6mM. The mixture was incubated for 60 min at 23°C with gentle swirling. The crosslinked species obtained were examined by SDS PAGE (Methods 2.21.1).

2.14.3 Crosslinking intact bacteria with dimethylimidates

This method was based on that described by Halpin et al. (1981). Exponentially growing bacteria (after approx. 5h growth) were harvested and washed once with TNM buffer, pH8.0 and resuspended in the same buffer ($0.3\text{g wet wt ml}^{-1}$). The crosslinker (0.1M; Methods 2.14.1) was added to give a final concentration of 6mM. The bacterial suspension was incubated for 60 min at 23°C with gentle swirling. In some experiments potassium phosphate buffer, pH7.5 was used instead of TNM buffer, pH8.0; in this case the same volume of the 0.1M dimethylimidate solution was added every 15 min over the 60 min incubation period.

2.14.4 Crosslinking a French press homogenate with DMSI or DMPI (dimethylpimelimidate)

Freshly harvested bacteria were resuspended in TNM buffer, pH8.0 to a final concentration of $0.5\text{g wet wt ml}^{-1}$. An appropriate volume of 0.1M DMSI or DMPI (Methods 2.14.1) was added to the bacterial suspension to give a final concentration of 6mM, after which the bacterial suspension was passed through the French press (Methods 2.9.2). The same volume of a 0.1M solution of DMSI or DMPI was added to the resulting homogenate which was passed through the French press again. When all the French pressing had been completed, a further volume of the 0.1M solution of DMSI or DMPI was added to the French press homogenate which was then incubated at 23°C for

60 min with gentle swirling. In experiments where the bacteria were resuspended in potassium phosphate buffer, pH7.5 instead of TNM buffer, pH8.0, the crosslinking of the French press homogenate was carried out as above but the same volume of the 0.1M solution of DMSI or DMPI was added every 15 min throughout the 60 min incubation period. Unbroken bacteria and debris were removed by centrifuging at 15,000g for 20 min at 4°C in an MSE 18 Highspeed centrifuge.

2.15 Crosslinking with phenylene dimaleimide (PDM)

2.15.1 Crosslinking intact bacteria with PDM

Exponentially growing bacteria (after approx. 5h growth) were harvested, washed once with 0.1M potassium phosphate buffer, pH7.5 containing 100µM thiamin pyrophosphate (TPP; cocarboxylase), 2mM MgSO₄ and 1mM EDTA and resuspended (0.3g wet wt ml⁻¹) in the same buffer. PDM was added to a final concentration of 100µM and the bacterial suspension was incubated at 23°C for 30 min. The reaction was terminated by the addition of dithiothreitol (DTT) to a final concentration of 200µM. The bacteria were washed once with 0.1M potassium phosphate buffer, pH7.5 containing 100µM DTT, 100µM TPP, 2mM MgSO₄ and 1mM EDTA and disrupted by sonication (Methods 2.9.1).

2.15.2 Crosslinking a French press homogenate with PDM

Freshly harvested bacteria were washed once with 0.1M potassium phosphate buffer, pH7.5 containing 100µM TPP, 2mM MgSO₄ and 1mM EDTA and resuspended (0.5g wet wt ml⁻¹) in the same buffer. PDM was added to the bacterial suspension to a concentration of 100µM before the bacteria were disrupted by two passages through the French pressure cell (Methods 2.9.2). When the French pressing was complete, more PDM was added and the samples were incubated at 23°C for 30 min. The reaction was terminated by the addition of DTT to a final concentration of 200µM. Unbroken bacteria and debris were removed by centrifuging at 15,000g for 20 min at 4°C in an MSE 18 Highspeed centrifuge.

2.16 Percoll density gradient centrifugation

2.16.1 Stock solutions

(a) Percoll

(b) 2.5M sucrose in 0.5M potassium phosphate buffer, pH7.5

(c) 0.25M sucrose in 0.05M potassium phosphate buffer, pH7.5

(d) Stock Percoll solution : a solution was prepared by adding 18ml of Percoll (a) to 2ml 2.5M sucrose in 0.5M potassium phosphate buffer, pH7.5 (b).

2.16.2 Method

A 20% Percoll solution was prepared by making 22.2ml of the stock Percoll solution (d) to 100ml. One ml of sample was layered onto 20ml of this 20% Percoll solution in a Ti60 polycarbonate centrifuge tube. The samples were then centrifuged for 30 min at $64,000g_{av}$ (30K rpm, Ti60 rotor) after which 1ml fractions were collected from the top of the self-generated gradient. Density gradient marker beads were included in each run in a separate tube to calibrate the density gradient formed.

2.17 Enzyme assays

Enzymes were generally assayed in plastic 1cm light-path cuvettes in a Unicam SP8/100 spectrophotometer with an in built chart recorder. The temperature of an external cuvette holder and of the spectrophotometer cuvette carriage was maintained at 27°C by an attached heating unit. The reaction components, except the substrate, were added to the cuvettes which were standing in the external cuvette holder. Micropipettes (Eppendorf Marburg Mikropipet, V.A. Howe and Co.Ltd., London W11) were used to dispense volumes less than 1ml. The components of the reaction mixture were mixed using 'plumpers' (Calbiochem, San Diego, California 92112). The reactions were initiated by the addition of substrate and mixing. Upto 4 cuvettes could be placed in the spectrophotometer and the absorbance measured sequentially at 10-15s intervals for upto 5 min by means of a programmed cuvette changer. Different volumes of enzyme samples were usually assayed

to check the linearity of the assay. Enzyme assays were all carried out in duplicate for each sample.

Enzyme units are defined as μmol substrate converted min^{-1} ; specific activities are given as units of enzyme per mg protein.

2.17.1 Mandelate dehydrogenases

LMDH and DMDH were assayed by following the phenazine methosulphate (PMS)-dependent reduction of 2,6-dichlorophenolindophenol (DCIP) at 600nm as described by Hills & Fewson (1983a) and Allison et al. (1985b). A decrease in A_{600} of 7.18 units was taken to correspond to the oxidation of $1\mu\text{mol}$ of substrate at pH7.5.

The enzyme was assayed in reaction mixtures containing (total volume 3ml):

200 μmol (2ml of 0.1M) potassium phosphate buffer, pH7.5

200nmol (0.1ml of 2mM) DCIP

1 μmol (0.1 of 10mM) PMS

10mg [0.1ml of 10% (w/v)] bovine serum albumin (BSA)

enzyme sample (usually 50 μl)

1.5 μmol (0.1ml of 15mM) L-mandelate or 6 μmol (0.1ml of 60mM)

D-mandelate adjusted to pH7.5, to initiate the reaction.

The same assay conditions were used to assay the LMDH of P.putida except that 12.5 μmol (0.1ml of 125mM) L-mandelate, adjusted to pH7.5, was added to initiate the reaction.

2.17.2 Lactate dehydrogenases

LDH was assayed in the same way as DMDH (Methods 2.17.1) except that 6 μmol (0.1ml of 60mM) D-lactate was used as substrate.

The assay procedure for LMDH activity (Methods 2.17.1) was modified to measure LLDH by replacing L-mandelate with 1.5 μmol (0.1ml of 15mM) L-lactate as substrate and lowering the amount of BSA in the cuvette from 10mg to 1mg in order to obtain maximal activity.

2.17.3 Direct assay for PGDC activity

PGDC activity was measured by following the disappearance of phenylglyoxylate at 334nm (Hegeman, 1966a). A decrease in A_{334} of 0.0272 units corresponded to the decarboxylation of 1 μ mol of phenylglyoxylate.

The enzyme was assayed in reaction mixtures containing (total volume 3ml):

500 μ mol (1ml of 0.5M) potassium phosphate buffer, pH6.0

30nmol TPP (0.1ml of 300 μ M in 0.25M potassium phosphate buffer, pH7.0)

enzyme sample (usually 0.1M)

7.5 μ mol (0.1ml of 75mM) phenylglyoxylate, adjusted to pH6.0, to initiate the reaction.

2.17.4 Indirect, aldehyde dehydrogenase linked, assay for PGDC activity

PGDC activity was measured by coupling the formation of benzaldehyde to the reduction of NAD, catalysed by yeast aldehyde dehydrogenase (Barrowman & Fewson, 1985). NAD reduction was measured at 340nm; an increase of 2.07 units corresponded to the decarboxylation of 1 μ mol of phenylglyoxylate.

The enzyme was assayed in reaction mixtures containing (total volume 3.0ml):

200 μ mol (2ml of 0.1M) potassium phosphate buffer, pH7.0

6 μ mol (0.1ml of 60mM) NAD

200nmol TPP (0.1ml of 2mM in 0.25M potassium phosphate buffer, pH7.0)

1 unit yeast aldehyde dehydrogenase (added in 0.1ml in 1mM DTT)

enzyme sample (usually 50 μ l)

15 μ mol (0.1ml of 150mM) phenylglyoxylate, adjusted to pH7.0, to initiate the reaction.

The activity of each batch of aldehyde dehydrogenase was checked by measuring the rate of reduction of NAD in the presence of 3mM benzaldehyde. It was also checked that aldehyde dehydrogenase was present in non-rate limiting concentrations for a given sample by measuring the rate of reduction of NAD in the presence of increasing concentrations of aldehyde dehydrogenase.

2.17.5 Indirect, alcohol dehydrogenase linked, assay for PGDC activity

This alternative indirect assay of PGDC activity was modified from the method described by Ullrich (1970) for pyruvate decarboxylase. Here PGDC activity was measured by coupling the formation of benzaldehyde to the oxidation of NADH catalysed by horse liver alcohol dehydrogenase.

The enzyme was assayed in reaction mixtures containing (total volume 3.0ml):

1mmol (2ml of 0.5M) potassium phosphate buffer, pH6.0

0.5 μ mol (0.1ml of 5mM) NADH

17 μ g horse liver alcohol dehydrogenase (0.1ml of a 0.5mg protein ml⁻¹ solution)

200nmol TPP (0.1ml of 2mM in 0.25M potassium phosphate buffer, pH7.0)

enzyme sample (usually 50 μ l)

15 μ mol (0.1ml of 150mM) phenylglyoxylate, adjusted to pH6.0, to initiate the reaction.

It was checked that the horse liver alcohol dehydrogenase was present in non-rate limiting concentrations for a given sample by measuring the rate of oxidation of NADH in the presence of increasing concentrations of alcohol dehydrogenase. Care was also taken to include a minus substrate blank to compensate for any NADH oxidase activity present in the sample.

2.17.6 BDH of A.calcoaceticus

BDH activity was measured by following the reduction of NAD at 340nm (Beggs & Fewson, 1977). An increase of 2.07 units corresponded to the oxidation of 1 μ mol of benzaldehyde.

The enzyme was assayed in reaction mixtures containing (total volume 3.0ml):

200 μ mol (2ml of 0.1M) sodium pyrophosphate buffer, pH9.0

6 μ mol (0.1ml of 60mM) NAD

50 μ mol (0.1ml of 0.5M) KCl

enzyme sample (usually 50 μ l)

0.3 μ mol (0.1ml of 3mM) benzaldehyde to initiate the reaction.

2.17.7 NAD(P) dependent BDH's of P.putida

NAD and NADP dependent BDH activities were measured by following the reduction of NAD(P) at 340nm (Hegeman, 1966a). An increase of 2.07 units corresponded to the oxidation of 1 μ mol of benzaldehyde.

The enzyme was assayed in reaction mixtures containing (total volume 3.0ml):

200 μ mol (2ml of 0.1M) Tris/HCl buffer, pH8.0

2.5 μ mol (0.1ml of 25mM) NAD or NADP

enzyme sample (usually 50 μ l)

3 μ mol (0.1ml of 30mM) benzaldehyde to initiate the reaction.

2.17.8 Mandelate racemase

This method was modified from that described by Hegeman (1966a).

Mandelate racemase activity was assayed as for DMDH activity (Methods 2.17.1) but in addition, a sufficient quantity (approx.3mg protein) of a 'wall + membrane' fraction of mutant strain C1005 which had been grown on complex media supplemented with D,L-mandelate (Methods 2.11.2) was added to give non-rate limiting amounts of LMDH. D-mandelate was added (0.1ml of 125mM) to initiate the reaction.

2.17.9 Catechol-1,2-dioxygenase

Catechol-1,2-dioxygenase activity was measured by following the production of cis, cis-muconate at 260nm in quartz cuvettes (Hegeman, 1966a). An increase in A₂₆₀ of 5.6 units corresponded to the conversion of 1 μ mol of catechol to cis, cis-muconate.

The enzyme was assayed in reaction mixtures containing (total volume 3.0ml):

200 μ mol (2ml of 0.1M) potassium phosphate buffer, pH7.5

4 μ mol (0.1ml of 40mM) EDTA

0.3 μ mol (0.1ml of 3mM) catechol (freshly prepared).

The reaction was initiated by addition of an enzyme sample (usually 10 μ l).

2.17.10 Isocitrate dehydrogenase

Isocitrate dehydrogenase (ICDH) activity was assayed by the method of Borthwick et al. (1984) by following the reduction of NADP at 340nm. An increase in A_{340} of 2.07 units corresponded to the oxidation of 1 μ mol of isocitrate.

The enzyme was assayed in reaction mixtures containing (total volume 3.0ml):

450 μ mol Tris/HCl buffer, pH7.5; 1.5 μ mol $MnCl_2$ (2ml of 225mM Tris/HCl buffer, pH7.5 containing 0.75mM $MnCl_2$)

1.2 μ mol (0.1ml of 12mM) NADP

7.5 μ mol (0.1ml of 75mM) isocitrate.

The reaction was initiated by the addition of an enzyme sample (usually 20 μ l).

2.17.11 NADH oxidase

NADH oxidase activity was measured by following the oxidation of NADH at 340nm (Kennedy & Fewson, 1968b). A decrease in A_{340} of 2.07 units corresponded to the oxidation of 1 μ mol of NADH.

The enzyme was assayed in reaction mixtures containing (total volume 3.0ml):

50 μ mol (0.5ml of 0.1M) sodium pyrophosphate buffer, pH7.0

500nmol NADH (0.1ml of 5mM in 0.05M sodium pyrophosphate buffer, pH7.0).

The reaction was initiated by the addition of an enzyme sample (usually 50 μ l).

2.17.12 Glutamine synthetase

Glutamine synthetase was assayed by the pyruvate kinase and lactate dehydrogenase coupled procedure described by Kingston et al. (1968). In this method, the ADP produced by glutamine synthetase is converted to ATP by reaction with phosphoenol pyruvate (PEP) catalysed by pyruvate kinase. The pyruvate thus produced is reduced by NADH in the presence of lactate dehydrogenase. The overall reaction was monitored by the oxidation of NADH at 340nm. A decrease in A_{340} of 2.07 units corresponded to the oxidation of 1 μ mol of NADH and thus to the formation of 1 μ mol of glutamine.

The enzyme was assayed in reaction mixtures containing (total volume 3.0ml):

150 μ mol imidazole.HCl buffer, pH7.0; 150 μ mol $MgCl_2$; 30 μ mol KCl; 120 μ mol NH_4Cl ; 300 μ mol glutamic acid (2ml of 75mM imidazole HCl, pH7.0 containing 75mM $MgCl_2$, 15mM KCl, 60mM NH_4Cl , 0.15M glutamic acid).

1 μ mol (0.1ml of 30mM) NADH

3 μ mol (0.1ml of 30mM) PEP

22.8 μ mol (0.1ml of 228mM) ATP

75 μ g pyruvate kinase (in 50 μ l)

150 μ g lactate dehydrogenase (in 50 μ l).

The reaction mixture was allowed to equilibrate at 30°C for 10 min during which time traces of ADP and pyruvate would be consumed and about 0.1 μ mol NADH would be oxidised. When a stable baseline was reached, an enzyme sample was added to initiate the reaction.

2.18 3-Deoxy-2-keto-octonic acid determinations

3-Deoxy-2-keto-octonic acid (KDO) was measured by the thiobarbituric acid method of Keleti & Lederer (1974) as described by McDougall et al. (1983),

2.18.1 Stock solutions

(a) 0.05M H_2SO_4

(b) acid periodate: 0.1M $NaIO_4$ in 0.2M HCl. This solution was stored in a dark bottle.

(c) sodium arsenite: 4% sodium arsenite (w/v) in 0.5M HCl

(d) thiobarbituric acid: a 0.6% (w/v) solution was made as required by dissolving in boiling distilled water. The solution was allowed to cool to room temperature before use.

(e) butan-1-ol/HCl [$\frac{v}{v}$ 95:5 (w/v)].

2.18.2 Method

Samples (0.2ml) and the standard KDO (0.323mg ml^{-1}) and 2-deoxy-ribose (0.26mg ml^{-1}) solutions were incubated with H_2SO_4 [(a); 0.2ml] for 20 min at

100°C. The samples were allowed to reach room temperature and 0.2ml acid periodate (b) was added and incubated for 10 min. Sodium arsenite [(c); 0.8ml] was then added and the samples were shaken until the yellow colour was discharged. Thiobarbituric acid [(d); 3.2ml] was added and after shaking, the solution was heated to 100°C for 10 min. After cooling, 4ml of butan-1-ol/HCl (e) was added and mixed. The samples were centrifuged at 1200g for 5 min in an MSE Mistral 2L centrifuge at 4°C and the upper layer was removed by a Pasteur pipette. The absorbance was measured at 508nm and 552nm. The molar absorption coefficients ($\text{l.mol}^{-1}\text{cm}^{-1}$) were 3.46×10^4 at 552nm and 6.88×10^3 at 508nm for KDO and 2.42×10^4 at 552nm and 2.81×10^4 at 508nm for 2-deoxyribose.

2.19 pH measurement

The pH values of most solutions were determined using a direct reading pH meter (Model 7010; E.I.L. Ltd., Cumbernauld, Glasgow G67 1AG) connected to a combined glass electrode (224; Probion Ltd., Glenrothes, Fife KY6 3AE). The pH values of small volumes were measured with a pH meter (Model PHM84; Radiometer, Copenhagen) fitted with an assembly containing microelectrodes.

2.20 Protein estimation

The method of Lowry et al. (1951) or Bradford (1976) was used to estimate the protein contents of samples using BSA as standard. Protein elution from columns was followed by measuring the A_{280} of the eluant.

2.21 Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS PAGE)

These methods are based on Davis (1964) as modified by Hayes and Wellner (1969) and by Lumsden and Coggins (1977).

2.21.1 Phosphate buffer method

2.21.1.1 Stock solutions

(a) 1M sodium phosphate buffer, pH6.5: a solution was prepared containing 81.0g $\text{Na}_2\text{HPO}_4 \cdot \text{l}^{-1}$ plus 59.3g $\text{NaH}_2\text{PO}_4 \cdot \text{l}^{-1}$, the pH was checked and adjusted if necessary.

(b) 20% (w/v) SDS: the solution was stored at 30°C.

(c) Acrylamide-bisacrylamide solution: 28g acrylamide and 0.735g NN methylene bisacrylamide were made to 100ml with distilled water. This solution was stored at room temperature and used within one month.

(d) Running buffer: a solution of 0.1M sodium phosphate buffer, pH6.5 containing 0.1% (w/v) SDS was prepared and stored at room temperature.

2.21.1.2 Gel plates

Glass plates (dimensions 9.5 x 20cm and 10 x 20cm) were washed in Decon 75 to ensure they were free of silicon grease, then rinsed with distilled water. The plates were assembled using 1.5mm teflon spacers and silicon grease and placed into a home-made gel-making manifold suitable for making upto 4 gels at a time.

2.21.1.3 Preparation of gels [5%(w/v)]

A solution was prepared containing (additions in this order): 35ml acrylamide-bisacrylamide stock solution (c); 141ml distilled water; 20ml 1M sodium phosphate buffer, pH6.5 (a); 1ml 20% SDS solution (b); 100µl N,N,N',N'-tetramethylethylenediamine (TEMED) and 150mg ammonium persulphate. The solution was de-gassed and poured into the gel-making manifold and 4 x 24 track teflon combs were placed on top. The poured gels were allowed to polymerise, stored at 4°C and used within one week.

2.21.1.4 Preparation and loading of samples

Protein solutions were mixed with the following dissolving buffer: 0.25ml 20% (w/v) SDS; 50µl 2-mercaptoethanol; 1.0ml glycerol; 50µl 1M sodium phosphate buffer, pH6.5; 0.1ml 0.5% (w/v) Bromophenol Blue; made to 5ml

with distilled water. A volume of dissolving buffer at least equal to the protein solution was used; the maximum sample size was 35 μ l per track. The dissolved protein samples were heated to 100°C for 5 min before loading onto the gel. Once the electrophoresis apparatus had been assembled and both reservoirs had been filled with running buffer, the samples were loaded onto the gel using a micropipette.

2.21.2 Electrophoresis conditions and sample running

The samples were electrophoresed at 80mA per slab gel which were cooled by circulating an ice/water slurry around the electrophoresis apparatus. The electrophoresis was stopped when the dye had almost reached the bottom of the gel. The gel was then stained for protein (Methods 2.21.3).

2.21.3 Protein staining

The gels were stained with 0.1% (w/v) Coomassie Brilliant Blue G-250 in methanol/acetic acid/H₂O (50:10:40 by volume) for 1h at 60°C. The gels were destained with methanol/acetic acid/H₂O (10:10:80 by volume) at 60°C.

2.21.4 Tris/HCl buffer method (discontinuous SDS slab gels)

2.21.4.1 Stock solutions

(a) A solution was prepared containing 36.6g Tris and 0.23ml TEMED, the pH was adjusted to pH8.8 with concentrated HCl and the volume was made to 100ml with distilled water and stored at room temperature.

(b) Electrode buffer: 6g Tris; 28.8g glycine and 10ml 20% (w/v) SDS were made to 2l with distilled water and stored at room temperature.

(c) Acrylamide-bisacrylamide solution: 28g acrylamide and 0.735g NN methylene bisacrylamide were made to 100ml with distilled water. This solution was stored at room temperature and used within one month.

(d) A solution was prepared containing 19.2ml 0.1M Tris/HCl buffer, pH6.8; 0.8ml 20% (w/v) SDS; and 0.05ml TEMED.

(e) 20% (w/v) SDS: the solution was stored at 30°C.

2.21.4.2 Gel plates

Gel plates were prepared as described in Methods 2.21.1.2.

2.21.4.3 Preparation of gels

(a) Separating gel [12.5% (w/v)]

A solution was prepared containing: 25ml solution (a) Tris/HCl, TEMED buffer, pH8.8; 89.3ml acrylamide-bisacrylamide solution (c) (the amount of solution (c) was adjusted as appropriate for lower percentage gels); 1ml 20% (w/v) SDS (e) and 82.2ml distilled water. This solution was degassed at room temperature before the addition of 150mg ammonium persulphate. The gel mixture was poured into the gel making manifold, overlayed with propan-2-ol and allowed to polymerise for 30 min. The layer of propan-2-ol was then washed off with distilled water and the top of the gels were blotted dry with filter paper.

(b) Stacking gel [5.9% (w/v)]

A solution was prepared containing: 17.5ml acrylamide-bisacrylamide solution (c); 10ml solution (d) containing Tris/HCl buffer, SDS, TEMED pH6.8 and 55ml distilled water. The solution was degassed before the addition of 150mg ammonium persulphate. The gel mixture was poured on top of the separating gel and 4 x 24 track teflon combs or 4 x single track combs (for prep.gels) were placed on top. The gels were left to polymerise for 30-60 min, stored at 4°C and used within one week.

2.21.4.4 Sample preparation

The sample buffer was prepared as follows: 1.5g SDS, 8g sucrose and 100µg pyronium Y was added to 10ml of 0.5M Tris/HCl buffer, pH8.8. The samples were mixed in the following way: 80µl of sample, 25µl 0.2M DTT and 10µl of sample buffer. The sample was denatured by heating to 100°C for 5 min. The maximum sample volume was 35µl per track (or 1.5ml for single track prep.gel) and all samples were loaded onto the gel using a micropipette.

2.21.4.5 Electrophoresis conditions and sample running

Conditions were as described in Methods 2.21.2.

2.21.5 Molecular mass calibration on SDS PAGE

Molecular mass estimations from SDS PAGE were made by calibrating gels with proteins of known M_r . A calibration kit (Pharmacia, Sigma or BDH) was used for this purpose and a typical standard curve obtained is shown (Figure 3A).

2.21.6 Gel scans

Gels were scanned using an LKB 2202 Ultrosan Laser Densitometer.

A typical gel scan of calibration proteins is shown in Figure 3B.

2.21.7 Photography

Gels were photographed by members of staff at the Medical Illustration Unit, University of Glasgow.

2.22 Immunoblotting method

This method is based on that described by Towbin et al. (1979) as modified by Batteiger et al. (1982).

2.22.1 Stock solutions

(a) Transfer buffer: a solution was prepared containing 0.19M glycine; 0.025M Tris; 0.02% (w/v) SDS and 20% (v/v) methanol.

(b). Blocking buffer: a solution of 0.02M Tris buffer, pH7.2 containing 0.15M NaCl and 0.5% (v/v) Tween 20 was prepared and stored at room temperature.

(c) First incubation buffer: a solution of 0.02M Tris buffer, pH7.2 containing 0.15M NaCl, 0.5% (v/v) Tween 20 and 5% (v/v) normal donkey serum was prepared as required.

(d) Second incubation buffer: a solution of 0.02M Tris buffer, pH7.2 containing 0.15M NaCl and 5% (v/v) normal donkey serum was prepared as required.

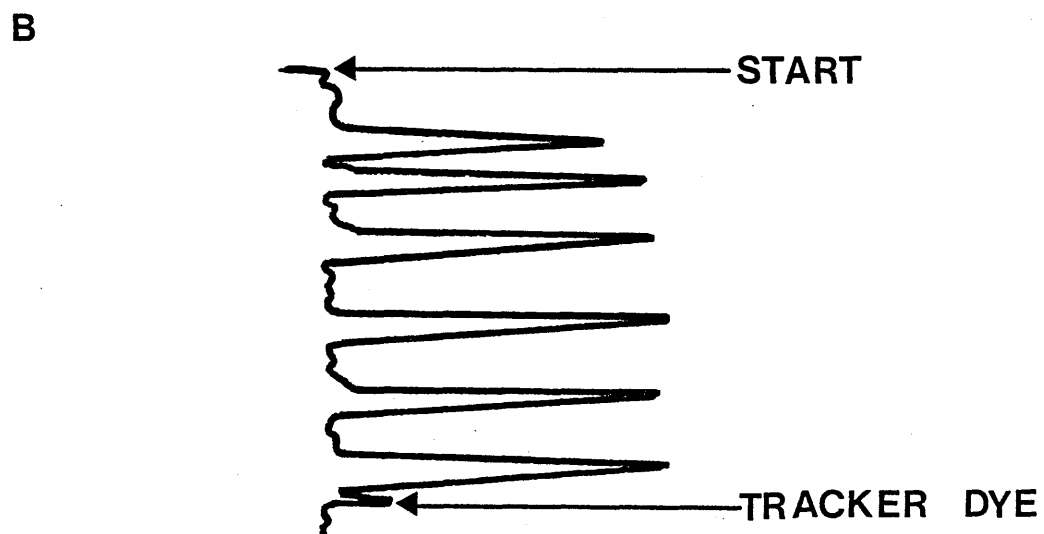
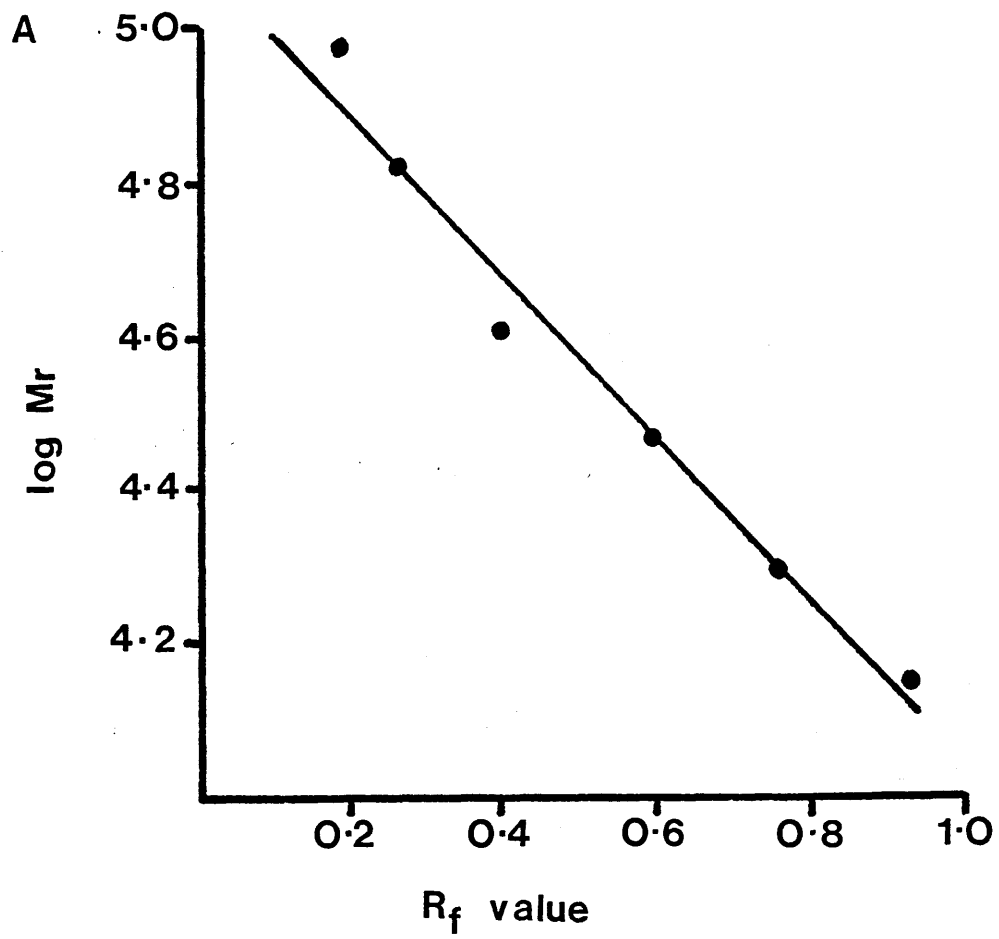
(e) Chloronaphthol stain: a solution of 30mg HRP colour development reagent in 10ml methanol, 50ml 10mM Tris buffer, pH7.2, 150 μ l 4% (v/v)

Figure 3 Standard curve of relative molecular mass calibration of
SDS PAGE

A sample of calibration proteins was subjected to SDS PAGE (Methods 2.21.4) on 12.5% gels. A standard plot of $\log_{10} M_r$ against R_f values allowed calibration of a set of SDS gels (A) and a typical gel scan (B) is shown.

The calibration proteins used were (from Pharmacia) -

Protein	M_r
Phosphorylase b	94,000
Bovine serum albumin	67,000
Ovalbumin	45,000
Carbonic anhydrase	30,000
Soybean trypsin inhibitor	20,100
α -Lactalbumin	14,400



H₂O₂ was mixed immediately before use.

2.22.2 Immunoblotting conditions

SDS PAGE was carried out (Methods 2.21.4). When electrophoresis was complete, the protein was transferred to 0.45µ nitrocellulose paper (Anderman and Co., Kingston Upon Thames, Surrey, KT2 6NH) using a Bio-Rad Trans-blot cell containing transfer buffer (a). The transfer was run at 350mA for 4h after which the nitrocellulose paper was placed in blocking buffer (b) and incubated at 4°C for at least 16h. The nitrocellulose paper was then transferred to the 'first incubation buffer' (c) to which the antibody of interest was added (usually a 1:200 dilution of the antibody). This was incubated at 23°C for 90 min after which the nitrocellulose paper was washed 4 x 12 min in 0.02M Tris buffer, pH7.2 containing 0.15M NaCl and 0.5% (v/v) Tween 20 and then washed 1 x 12 min in 0.02M Tris buffer, pH7.2 containing 0.15M NaCl. The nitrocellulose paper was placed in the 'second incubation buffer' (d) to which a 1:1000 dilution of HRP donkey anti-rabbit IgG was added and incubated at 23°C for 90 min. The nitrocellulose paper was washed 5 x 12 min in 0.02M Tris buffer, pH7.2 containing 0.15M NaCl, after which it was placed in the chloronaphthol stain (e) for approx. 2 min and then washed in excess distilled water. The nitrocellulose paper was then dried and stored.

2.23 Column chromatography

2.23.1 Apparatus

The glassware and adaptors of the columns used were obtained from LKB Instruments Ltd. and Pharmacia Ltd. Solutions were pumped through the column using a 'varioperpex' peristaltic pump (LKB Instruments Ltd.) and the eluants collected with an automatic fraction collector (Model 7000 Ultrarac, LKB Instruments Ltd.).

2.23.2 Column chromatography media

All column chromatography material was prepared and treated as advised by the instruction booklet of the appropriate manufacturer.

2.23.3 Relative molecular mass estimations from gel filtration

Molecular mass estimations were made by gel filtration on columns calibrated with proteins of known molecular mass.

2.24 Purification of LMDH

All steps in the purification procedure were carried out at 4°C.

2.24.1 Preparation of a 'wall + membrane' fraction

Frozen bacteria [70g of mutant strain C1005 which had been grown on complex medium plus D,L-mandelate (Methods 2.7.2)] were resuspended in 0.05M potassium phosphate buffer, pH7.5 (0.5g wet wt ml⁻¹) and were disrupted by four passages through a French pressure cell (Methods 2.9.2). The resulting homogenate was centrifuged at 165,000g_{av} (40K rpm, Ti60 rotor) for 150 min and the pellet was washed twice by resuspension/centrifugation (Methods 2.11.2) and resuspended to a final volume of 70ml. This fraction was either used immediately or stored at -20°C.

2.24.2 Preparation of Triton extract

The 'wall + membrane' fraction was diluted to 20mg protein ml⁻¹ and 10% (w/v) Triton X-100 was added to a final concentration of 0.5mg of detergent per mg of protein. The suspension was stirred gently for 30 min and then centrifuged at 165,000g_{av} (40K rpm, Ti60 rotor) for 120 min. Ethanediol was added to the supernatant ('Triton extract') to a final concentration of 20% (v/v) and the Triton extract was then either used immediately or stored at -20°C.

2.24.3 Ion exchange chromatography I

The Triton extract was applied to a column of DEAE-Sephacel (20cm long x 5.2cm diam.) which had been equilibrated with 0.02M potassium phosphate buffer, pH7.5 containing 0.1% (w/v) Triton X-100, 100µM DTT and 20% (v/v) ethanediol. The column was washed (2-3 column volumes) with the same buffer containing 0.1M NaCl to remove unbound material. Protein was eluted by applying a linear gradient of 0.1-0.25M NaCl in the same buffer (total volume 2l) at 60ml h⁻¹ and fractions were collected at 6 min intervals.

The A_{280} of the eluent was monitored and fractions were assayed for LMDH activity. Conductivities were measured with a radiometer (Copenhagen, Denmark) type CDM 2e conductivity meter.

2.24.4 Ammonium sulphate fractionation

Solid $(\text{NH}_4)_2\text{SO}_4$ (specially low in heavy metals for enzyme work) was added to the pooled ion exchange fractions to give 15% saturation. When all the $(\text{NH}_4)_2\text{SO}_4$ had dissolved, the solution was stirred for 30 min before centrifuging at $65,000g_{\text{av}}$ (25K rpm, 10 x 100ml PrepSpin rotor) for 30 min at 4°C . More $(\text{NH}_4)_2\text{SO}_4$ was added to the resulting supernatant to give 30% saturation and the solution was again stirred at 4°C for 30 min and centrifuged as before. The floating material was then resuspended in 0.02M potassium phosphate buffer, pH7.5 containing 0.05% (w/v) Triton X-100, 100 μM DTT and 10% (v/v) ethanediol.

2.24.5 Gel filtration chromatography

The 15-30% $(\text{NH}_4)_2\text{SO}_4$ fraction of the ion exchange pool was applied to a column of Ultrogel AcA-34 (71cm long x 5.2cm diam.) which had been equilibrated with 0.02M potassium phosphate buffer, pH7.5 containing 0.05% (w/v) Triton X-100, 100 μM DTT and 10% (v/v) ethanediol. Protein was eluted with the same buffer at 60ml h^{-1} and fractions were collected every 10 min. Fractions containing LMDH activity were pooled.

2.24.6 Ion exchange chromatography II

Pooled fractions from the gel filtration column were applied to a second column of DEAE-Sephacel (5cm long x 2.6cm diam.) pre-equilibrated with 0.02M potassium phosphate buffer, pH7.5 containing 0.05% Triton X-100, 100 μM DTT and 10% (v/v) ethanediol. The column was washed (2-3 column volumes) with the same buffer containing 0.1M NaCl. A linear gradient of 0.1-0.25M NaCl was applied (total volume 400ml) at 20ml h^{-1} and fractions were collected every 6 min. Fractions containing LMDH activity were pooled, ethanediol was added to a final concentration of 20% (v/v) and the pool was stored at -20°C .

2.25 Analysis of flavin in purified enzyme

The flavin content of LMDH was analysed as described by Allison *et al.* (1985b). This method involves extraction of non-covalently bound flavin by trichloroacetic acid (TCA). After addition of the ice cold TCA [final concentration 10% (v/v)], the solution was incubated at 4°C for 15 min before centrifuging at 15,000g for 10 min. The protein precipitate was re-extracted with TCA in the same way. The combined extracts (supernatants) were extracted twice with 4 volumes of diethylether to remove the TCA. Residual diethylether was removed from the aqueous layer under a stream of N₂. The extract was neutralised by addition of 0.5ml of 1M potassium phosphate buffer, pH7.5 and made to a final volume of 3ml with 0.1M potassium phosphate buffer, pH7.5. The emission spectrum of the sample in a 3ml quartz cuvette was recorded in a Perkin-Elmer 3000 fluorescence spectrophotometer (exciting at 450nm) and compared with standard solutions of FAD and FMN.

2.26 Raising antiserum to LMDH

The recovery of LMDH at the final stage of the purification was poor and so a modified version of the method of Mikara & Blobel (1980) was used to produce protein for injection. In this method a less purified sample may be used by first running on a SDS polyacrylamide gel and cutting out the band corresponding to the enzyme.

2.26.1 Concentration of gel filtration pool

The pooled fractions from the gel filtration column (Methods 2.24.5) were treated with (NH₄)₂SO₄ (Methods 2.24.4) to yield a 15-30% saturated fraction which was resuspended in 5-10ml of 0.02M potassium phosphate buffer, pH7.5 containing 0.05% (w/v) Triton X-100, 100μM DTT and 10% (v/v) ethanediol.

2.26.2 Preparing LMDH for injection

The 15-30% (NH₄)₂SO₄ fraction (1.5ml) was applied to a single track 12.5% SDS gel (Methods 2.21.4). The gel was then stained with Coomassie

Blue (Methods 2.21.3) and destained (Methods 2.21.3).

The band corresponding to LMDH was cut out of the gel and washed in distilled H_2O until the pH of the wash was 7.0. The gel was ground in liquid N_2 and the powder was immediately transferred to the barrel of a 2ml syringe. Distilled H_2O (0.5ml approx.) was added and the sample was sonicated for 3 x 30 sec using the microtip probe of the Dawe Soniprobe (Type 7332A; Dawe Instruments Ltd., London). An equal volume of Freund's complete adjuvant was added and the sonication was repeated. The emulsion was then injected into 1ml syringes. Subsequent samples were prepared with Freund's incomplete adjuvant.

2.26.3 Injection of rabbit and preparation of serum

This stage was carried out by Dr. I.D. Hamilton. A rabbit weighing 4kg (approx.) was injected subcutaneously at several sites with the equivalent of 100 μ g (total) of LMDH. After 5 weeks a further 100 μ g was injected and a booster injection was given 2 weeks later. After a further two weeks, 50ml (approx.) of blood was collected from the rabbit's ear vein.

Blood samples were allowed to clot at room temperature and left overnight at 4°C. The serum was pipetted off and centrifuged at 15,000g for 10 min to remove red blood cells. One ml samples were stored at -20°C.

2.26.4 Adsorption of LMDH antiserum

A sonic extract (Methods 2.9.1) (10mg protein ml^{-1}) of strain 8250 grown on 5mM succinate/salts medium (Methods 2.7.3) (i.e. containing no detectable LMDH activity) was denatured by boiling in the presence of 1.3% (w/v) SDS and 45mM DTT for 5 min. An equal volume of this denatured extract was incubated with LMDH antiserum for 4h at 23°C after which the sample was centrifuged in an Eppendorf 3200 centrifuge for 5 min. The supernatant (adsorbed antiserum) was stored in 1ml samples at -20°C.

2.27 Antibody enzyme inhibition studies

Appropriate concentrations of enzyme extracts (100 μ l) were incubated with appropriate dilutions of antisera (100 μ l) for 1h at 23°C. Enzyme samples were also incubated with buffer or normal rabbit serum as controls. The samples were centrifuged for 5 min in an Eppendorf 3200 centrifuge and enzyme activities were measured in the supernatants.

2.28 Immunoprecipitation studies

Appropriate concentrations of enzyme extracts (100 μ l) were incubated with undiluted antisera (100 μ l) or with normal rabbit serum or buffer as controls for 1h at 23°C. Protein A-Sepharose (100 μ l) was added and the samples were incubated for a further hour at 23°C before immunocomplexes were precipitated by centrifuging for 5 min in an Eppendorf 3200 centrifuge.

2.29 Statistical methods

Means and standard deviations, correlation coefficients (r) and the Students t-test were calculated using a Casio fx-180P calculator (Casio Computer Co. Ltd.). All results are quoted as means \pm standard deviations with the number of determinations in parentheses.

2.30 Determination of kinetic constants

The Michaelis constant of IMDH for L-mandelate was determined by measurement of initial velocities of enzyme activities with a range of substrate concentrations. The Enzpack computer programme (Williams, 1985) was used to calculate the K_m by the 'direct linear' method.

2.31 Glassware

2.31.1 General glassware

All growth flasks were cleaned before use by autoclaving in Hemosol solution (approx 10g l⁻¹, Meinecke and Co., Baltimore, USA). The glassware was thoroughly rinsed with tap water and then with distilled water before drying in an oven. Other glassware was washed in Hemosol, rinsed and dried.

2.31.2 Pipettes

All pipettes were cleaned by soaking in 'Kirbychlor' disinfectant solution (H. and T. Kirby and Co. Ltd., Meldenhall, Suffolk) then in Hemosol solution followed by thorough rinsing in tap and deionised water and drying in an electrically heated pipette drier. All pipettes were plugged with non-absorbant cotton wool.

2.32 Safety

Bacterial cultures were killed by autoclaving before disposal. Any bacterial spillage was swabbed with 10% (v/v) propan-1-ol. All other precautions taken in the interest of safety were as described in the University of Glasgow Safety Handbook.

CHAPTER 3 RESULTS

Section 3.1

Separation of outer and inner membranes of

A.calcoaceticus and location of lactate and
mandelate dehydrogenases

3.1.1 Introduction

Although the mandelate and lactate dehydrogenases of A.calcoaceticus strain NCIB 8250 have been designated as membrane-bound (Fewson & O'Donnell, 1981; Hills & Fewson, 1983a) it was not known whether these enzymes are inner or outer membrane proteins. Miura & Mizushima (1968) first demonstrated the separation of outer and inner membranes of E.coli after treating the bacteria with lysozyme. Subsequently, methods have been developed combining French press disruption with lysozyme treatment and sucrose density gradient centrifugation (Schnaitman, 1970; Thornley et al., 1973). Purified outer membranes have also been obtained by selectively solubilising the cytoplasmic membrane with detergents (De Pamphilis & Adler, 1971 a and b; Schnaitman, 1971). Since active enzymes were required in this present study, these latter methods involving digestion of the cytoplasmic membrane were not suitable.

Previous attempts to achieve lysis of A.calcoaceticus NCIB 8250 by lysozyme had been unsuccessful (personal communications Prof. C.A. Fewson; Dr. L.M. Fixter). Separation of outer and inner membranes of Acinetobacter has been reported in strain H01-N after lysozyme treatment (Scott et al., 1976) and in strain MJT/FS/199A by a combination of French press disruption and lysozyme treatment (Thornley et al., 1973). However, this latter strain is oxidase positive and should probably be excluded from the genus Acinetobacter (McDougall et al., 1983). Nonetheless, it was thought that a combination of these methods might prove useful.

3.1.2 Separation of outer and inner membranes of A.calcoaceticus NCIB 8250

Preliminary attempts to separate outer and inner membranes of A.calcoaceticus NCIB 8250 by combining French press disruption with lysozyme treatment as described by Thornley et al. (1973) were unsuccessful and so a method for lysozyme treatment of A.calcoaceticus

NCIB 8250 was developed based on that described by Scott et al. (1976).

a) Time course for lysozyme treatment of strain Cl219

Freshly harvested bacteria were incubated with lysozyme (Methods 2.9.3.1) and samples were taken immediately and at various time intervals upto 150 min. The OD₅₀₀ and the ICDH activity released on diluting the samples in the absence and presence of 0.6M sucrose was measured (Figure 4). ICDH is a cytoplasmic enzyme and therefore if any activity is released this indicates that disruption of bacteria has taken place.

Addition of lysozyme to the bacterial suspension had an immediate effect. Approx. 50% of the total cell lysis (based on ICDH released when the samples were diluted in cold distilled water) took place virtually immediately (Figure 4B). Since there was very little change in the OD₅₀₀ and since no ICDH was released when the samples were diluted in buffer containing 0.6M sucrose, it can be concluded that the spheroplasts formed as a result of lysozyme treatment are stable over the 150 min incubation period. Similar time course profiles were obtained for other mutant strains of A.calcoaceticus derived from the wild type strain NCIB 8250 (results not shown).

b) Investigation of centrifugation conditions required to separate outer and inner membranes

Attempts were made to separate the outer and inner membranes; NADH oxidase was used as a marker for the inner membrane and KDO was used as an outer membrane marker (Figure 5A).

The outer membrane remained in the supernatant and most of the inner membrane sedimented under the conditions of centrifugation used (Figure 5A); upto 70% NADH oxidase was recovered in the pellet whereas 95% KDO remained in the supernatant. The supernatant (outer membrane fraction) was contaminated with 30% of the inner membrane whereas the pellet fraction (inner membrane) showed little contamination (5%) by the outer membrane. More inner membrane was sedimented when the sample was centrifuged for

Figure 4 Time course for lysozyme treatment of mutant strain C1219

Bacteria (mutant strain C1219) were treated with lysozyme (10mg per g wet wt) in the presence of 0.05M Tris/HCl buffer, pH7.8 containing 0.6M sucrose (Methods 2.9.3.1). Samples were taken over the 150 min incubation period and were diluted in cold distilled water (—) or in 0.05M Tris/HCl buffer, pH7.8 containing 0.6M sucrose (----). The OD₅₀₀ (A) of the bacterial suspension was measured before (●) and after (○) the addition of lysozyme. The ICDH activity (B) in the supernatants, obtained when the diluted samples were centrifuged for 5 min in an Eppendorf 3200 centrifuge, was measured before (▲) and after (△) the addition of lysozyme.

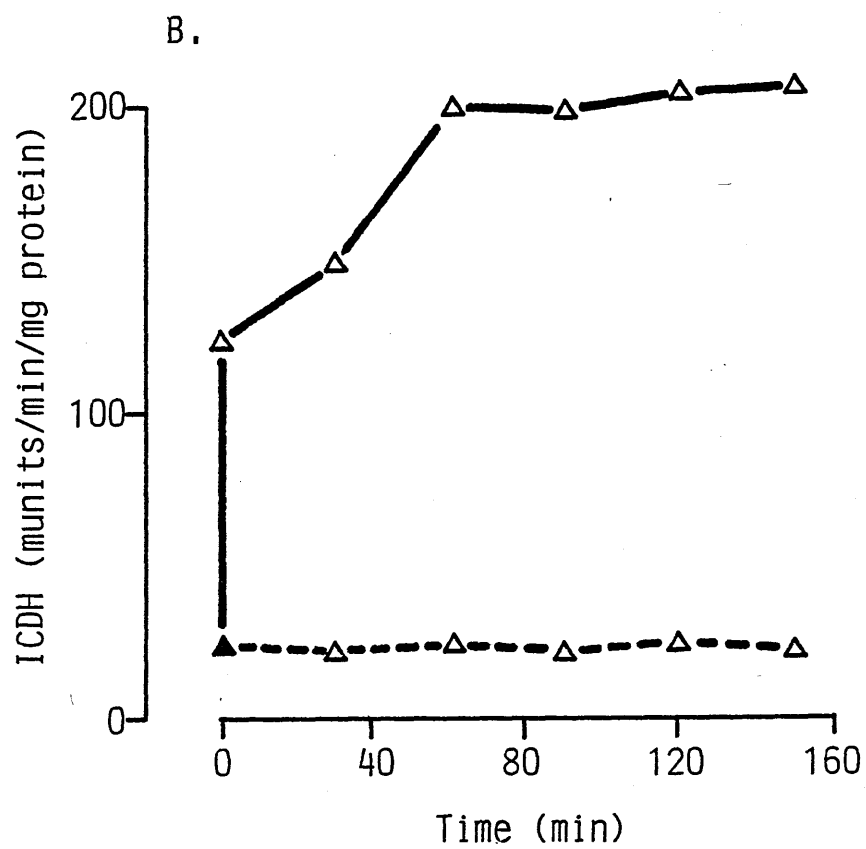
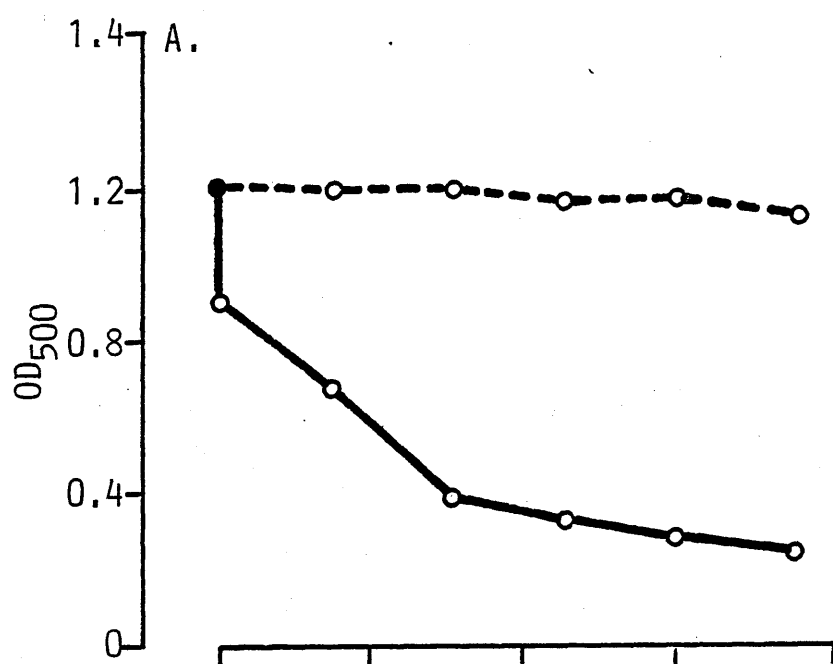


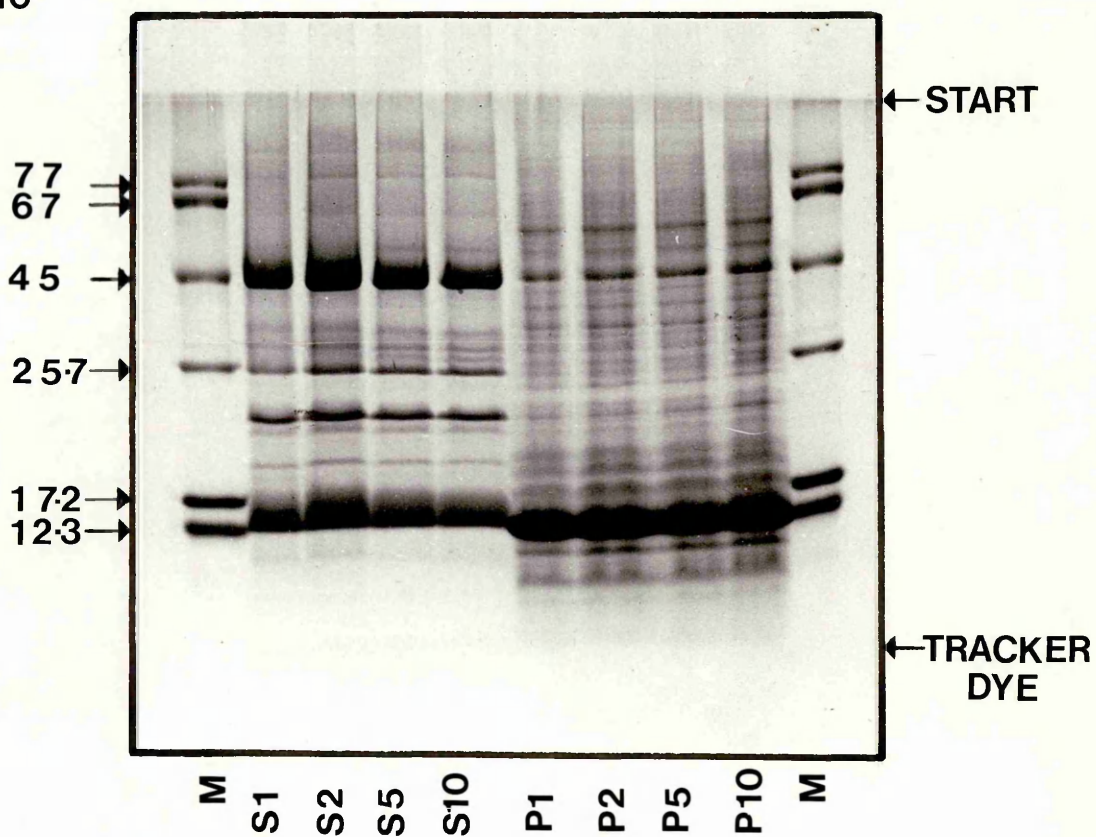
Figure 5 Optimising the centrifugation times for separation of outer and inner membranes

Freshly harvested bacteria (mutant strain C1219) grown on complex media) were treated with lysozyme (Methods 2.9.3.1) and a crude membrane fraction was prepared (Methods 2.10) and centrifuged in an Eppendorf 3200 centrifuge for various times. The pellets and supernatants were assayed for NADH oxidase, KDO and protein (A) and subjected to SDS PAGE on a 10% gel (Methods 2.21.4). Approx. 50µg protein was loaded on each track. The gel was stained with Coomassie Blue (B) and standard proteins of known molecular mass (M) obtained from BDH Chemicals Ltd. were used to calibrate the gel:- ovotransferrin, M_r 77,000; albumin, M_r 67,000; ovalbumin, M_r 45,000; chymotrypsinogen A, M_r 25,700; myoglobin, M_r 17,200 and cytochrome c, M_r 12,300. S = supernatant (outer membrane); P = pellet (inner membrane). Numbers refer to time of centrifugation (min).

A.

Centrifugation time (min)	Fraction	NADH oxidase (% of total)	KDO (% of total)	Protein
1	supernatant	35	98	80
	pellet	65	2	20
2	supernatant	36	98	80
	pellet	64	2	20
5	supernatant	36	95	74
	pellet	64	5	26
10	supernatant	30	95	76
	pellet	70	5	24

B.

 $M_r \times 10^{-3}$


longer than 10 min, however, this resulted in a greater contamination of the inner membrane by the outer membrane (results not shown).

c) SDS PAGE of outer and inner membranes

The membrane fractions were analysed by SDS PAGE (Figure 5B). The outer and inner membranes showed different patterns on SDS PAGE although the patterns obtained were not altered by varying the time of centrifugation. The outer membrane fractions (supernatants) were dominated by four major proteins with apparent M_r values of 44,000, 28,000, 22,000 and 13,000. Cytoplasmic (inner) and outer membranes were separated in subsequent experiments by centrifuging for 10 min (Methods 2.10).

d) Analysis of outer and inner membrane fractions by electron microscopy

The outer and inner membrane fractions were examined by electron microscopy using the negative staining technique (Methods 2.13). The outer membrane fractions were a homogeneous suspension of open 'C' structures (Figure 6A) whereas the cytoplasmic membrane fractions were composed of larger fragments and vesicles of various sizes (Figure 6B).

3.1.3 Location of DMDH, LMDH, DLDH and LLDH

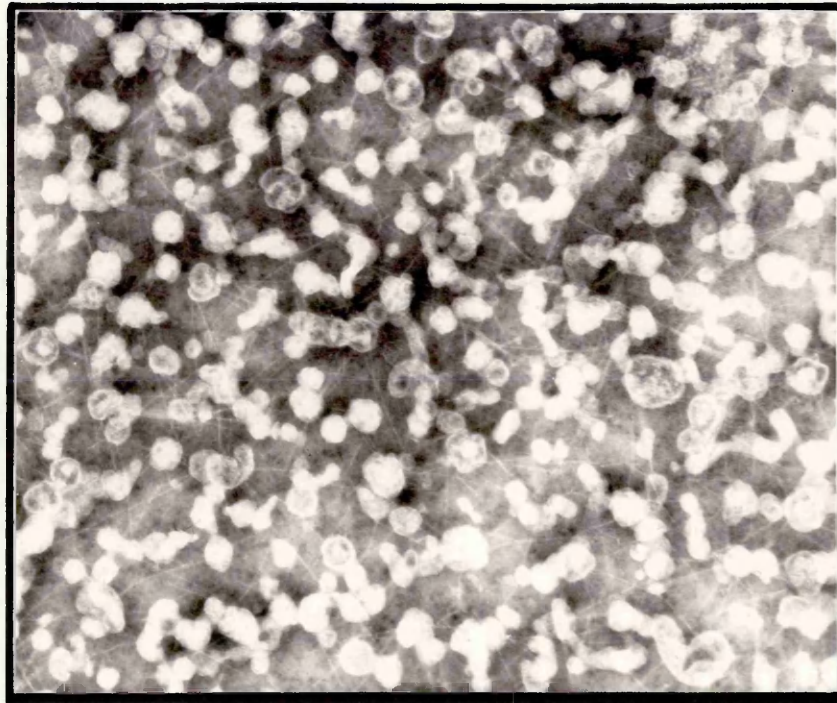
Outer and inner membrane fractions were prepared from mutant strain Cl219. Each fraction was assayed for the four dehydrogenases - DMDH, LMDH, DLDH and LLDH as well as for NADH oxidase and KDO (Table 2).

All four dehydrogenases showed the same pattern as NADH oxidase in terms of both percentage distribution and specific activities (Table 2); 65-75% of the enzymes were located in the inner membrane fraction and so it can be concluded that DMDH, LMDH, DLDH and LLDH are cytoplasmic membrane proteins.

Figure 6 Electron micrographs of outer and inner membranes

Membrane fractions were negatively stained with 1.5% phosphotungstic acid, pH7.0 (Methods 2.13). After incubating at room temperature for 1 min, excess fluid was removed and the grids were air dried. A = outer membrane. B = inner membrane.

A



0.5 μ m

B

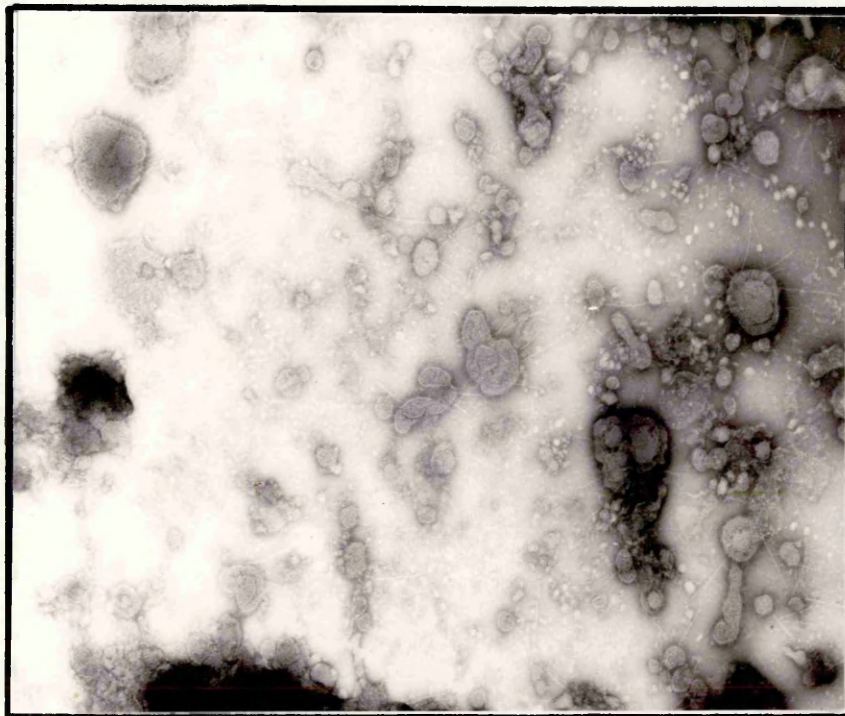


Table 2 Distribution of lactate dehydrogenases and mandelate dehydrogenases in the outer and inner membranes

Component	Specific activity (munits per mg protein)		% of total enzyme units KDO or protein	
	inner membrane	outer membrane	inner membrane	outer membrane
LMDH	1078	358	66	34
DMDH	252	59	74	26
LLDH	469	151	67	33
DLDH	890	281	68	32
NADH oxidase	183	66	65	35
KDO	5.4 μ g ml ⁻¹	104 μ g ml ⁻¹	5	95
Protein	3.1 mg ml ⁻¹	5.08 μ g ml ⁻¹	40	60

Bacteria (mutant strain C1219 which had been grown on complex media supplemented with D,L-lactate and D,L-mandelate) were treated with lysozyme (Methods 2.9.3.1) and outer and inner membrane fractions were prepared (Methods 2.10). Each fraction was assayed for LMDH, DMDH, LLDH, DLDH, NADH oxidase, KDO and protein and the results were expressed in terms of both specific activities and percentage of totals.

3.2.1 Introduction

Many preliminary experiments attempting to purify LMDH were carried out in conjunction with the work on the purification of DMDH and DLDH by Drs. M.J. O'Donnell and N. Allison (also Allison et al., 1985b).

Neither LMDH nor LLDH had been purified to homogeneity, partly because of problems resulting from their sensitivity to high salt concentrations and low pH values.

3.2.2 Preliminary experiments on LMDH purification

LMDH was taken through purification stages which were based on those for DMDH and DLDH (Allison et al., 1985b) but modified accordingly. LMDH was solubilised from a 'wall + membrane' fraction by Triton X-100 extraction (Methods 2.24.2) and the resulting Triton extract was treated with $(\text{NH}_4)_2\text{SO}_4$ (Methods 2.24.4). The 15-30% saturation $(\text{NH}_4)_2\text{SO}_4$ fraction was resuspended in 0.02M potassium phosphate buffer, pH7.5 containing 0.1% (w/v) Triton X-100, 100 μM DTT and 10% (v/v) ethanediol and applied to a 1.4 l gel filtration column (71cm long x 5.2cm diam.) of Ultrogel AcA 34 which had been equilibrated with the same buffer. Fractions containing LMDH activity were pooled and applied to a column (15cm long x 2.6cm diam.) of DEAE Sephacel which had been equilibrated with 0.02M potassium phosphate buffer, pH7.5 containing 0.05% Triton X-100, 100 μM DTT and 20% (v/v) ethanediol. The column was washed (1-2 column volumes) with the same buffer before LMDH activity was eluted by a linear gradient of 0-250mM NaCl (total volume 500ml) at a flow rate of 20ml h⁻¹.

The pooled fractions from the ion exchange column were dialysed against 0.02M piperazine.HCl buffer, pH5.7 containing 0.05% Triton X-100, 100 μM DTT and 5% (w/v) ethanediol. This dialysis resulted in a loss of 50% of the LMDH activity. The LMDH sample was applied to the Pharmacia fplc Mono P chromatofocusing column which had been equilibrated with the same piperazine buffer. LMDH activity was eluted from the column by a 1:8 dilution of Polybuffer 74, pH3.6 containing 0.05% Triton X-100, 100 μM DTT

and 5% (v/v) ethanediol at a flow rate of 60ml h^{-1} . The pH of the fractions collected was immediately raised by the addition of $100\mu\text{l}$ of 1M potassium phosphate buffer, pH7.5. There was poor recovery (20%) of LMDH from this column and this may have been due to the instability of the enzyme at its isoelectric point.

The purification was monitored by SDS PAGE (Figure 7). It can be seen that unlike the DMDH and DLDH, this purification scheme did not result in a homogeneous preparation of LMDH. Three major protein bands were observed in the Mono P pool and these have apparent M_r values of 66,000, 44,000 and 27,000. The possibility that LMDH is a multicomponent enzyme such as succinate dehydrogenase (Hederstedt & Rutberg, 1981) was discounted in preliminary experiments which showed that it was possible to separate these three proteins to some extent.

3.2.3 LMDH purification scheme

Since chromatofocusing proved unsuitable for purifying LMDH, it was necessary to investigate other methods for the final stage of the purification. The order of the purification scheme was altered so that gel filtration became the penultimate step thus avoiding the need to dialyse the sample at this stage.

a) Triton X-100 extraction

A 'wall + membrane' fraction was prepared (Methods 2.11.2) from 70g of frozen bacteria, mutant strain C1005 grown on complex media supplemented with D,L-mandelate (Methods 2.7.2), and the protein concentration was adjusted to 20mg ml^{-1} . LMDH was solubilised by Triton X-100 (Methods 2.24.2); 60-70% of the starting LMDH activity was solubilised in this way and a 3-4 fold purification was achieved.

b) Ion exchange chromatography I

The Triton X-100 extract was applied to a column of DEAE Sephacel (Methods 2.24.3) and the elution profile is shown in Figure 8. The recovery from the column was 30-50% and a 3 fold purification was achieved.

Figure 7 Gel scans of an SDS polyacrylamide gel of the ion exchange and Mono P LMDH pools

The pooled fractions containing LMDH activity from an ion exchange column and that from the fplc Mono P column (see text for details) were subjected to SDS PAGE (Methods 2.21.4) on 10% gels. Proteins of known molecular mass (from Sigma) were used to calibrate the gel:- phosphorylase b, M_r 97,400; albumin, M_r 67,000; ovalbumin, M_r 45,000; carbonic anhydrase, M_r 30,000; trypsin inhibitor, M_r 20,100. The gels were stained with Coomassie Blue and scanned (Methods 2.21.6).

- A. gel scan of the ion exchange LMDH pool (approx. 50 μ g protein loaded).
- B. gel scan of the Mono P LMDH pool (approx. 10 μ g protein loaded).

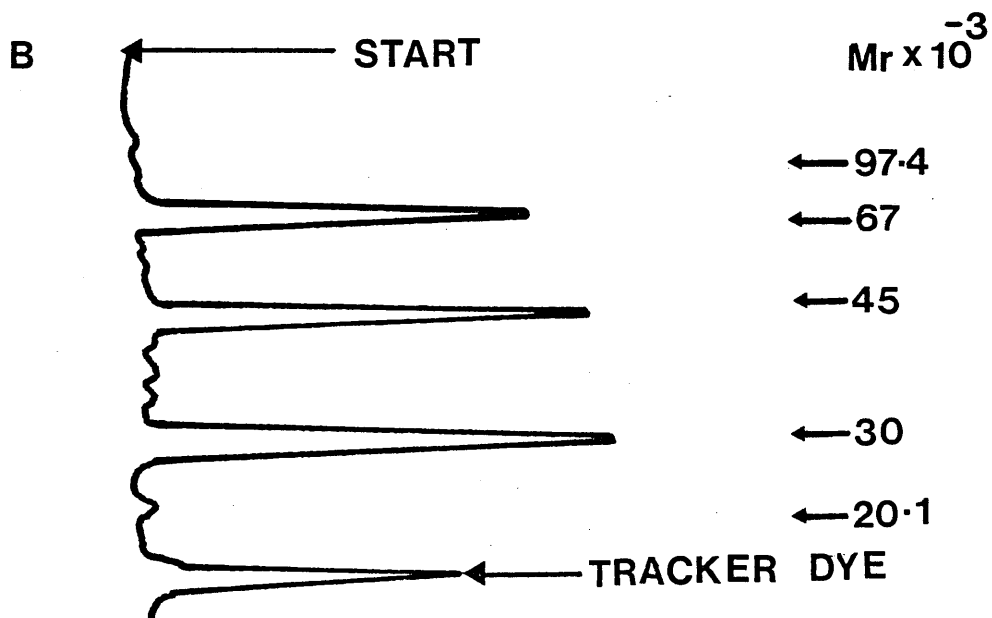
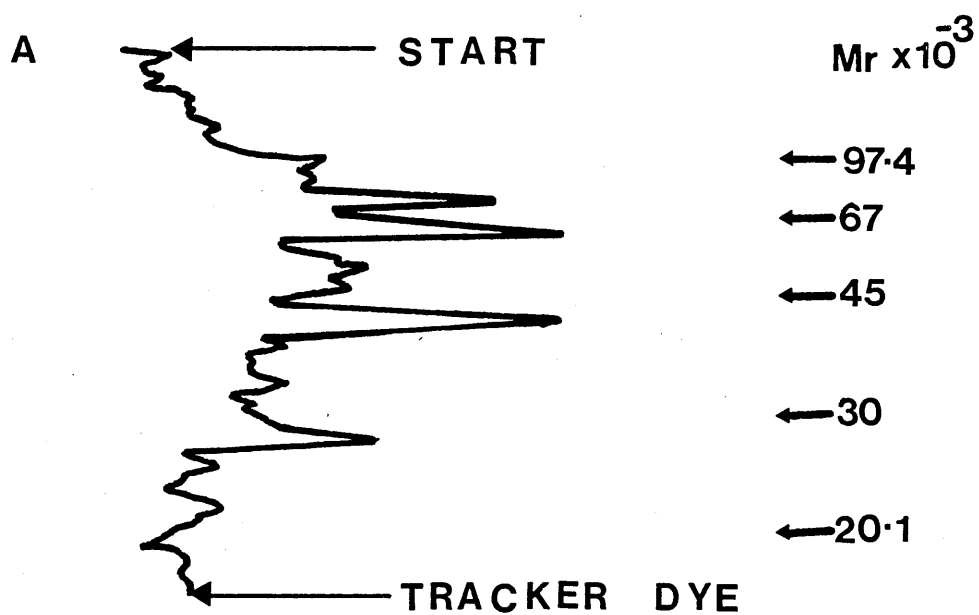
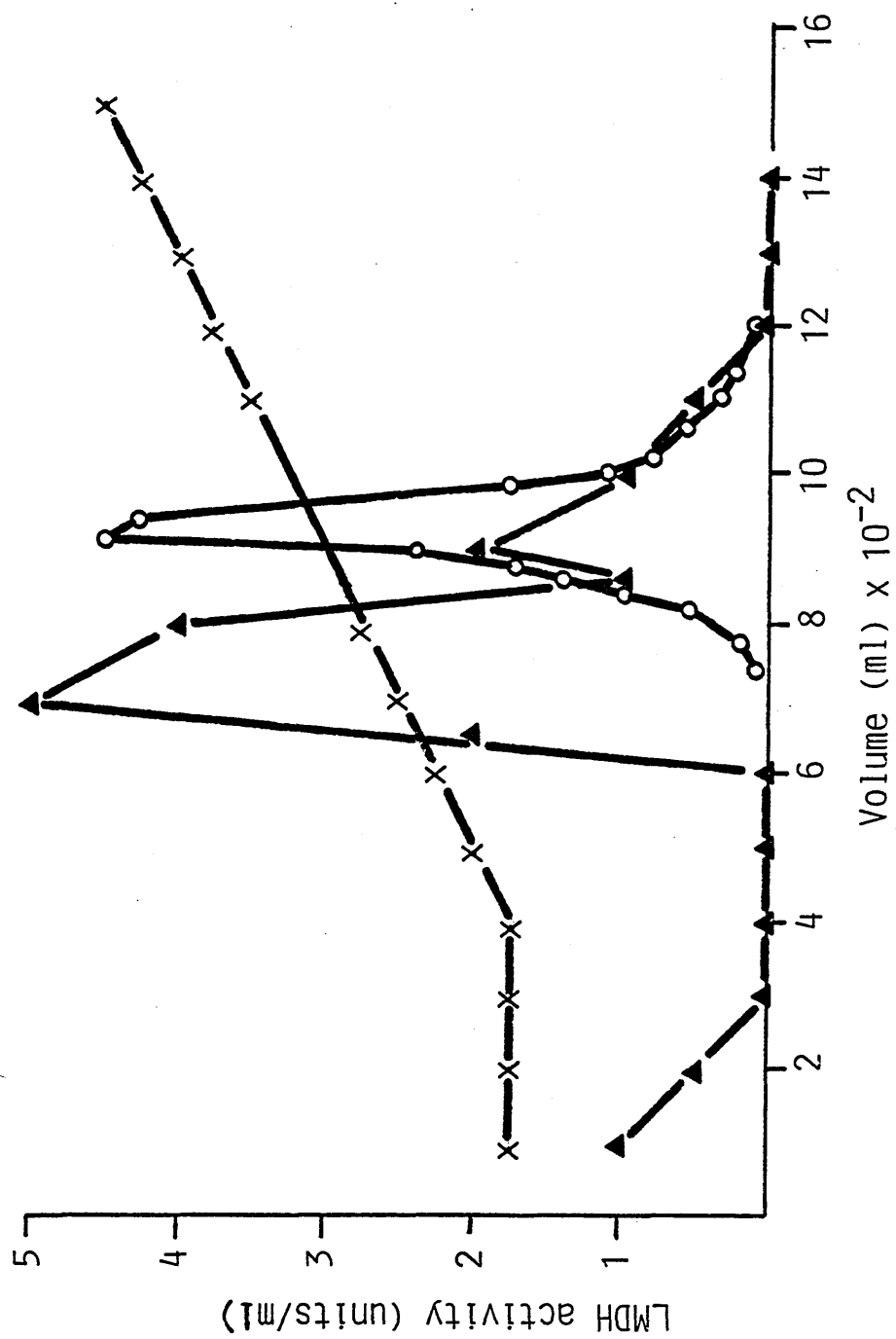


Figure 8 Ion exchange chromatography I of LMDH

A Triton X-100 extract (Methods 2.24.2) was applied to an ion exchange column (20cm long x 5.2cm diam.) of DEAE Sephacel which had been equilibrated with 0.02M potassium phosphate buffer, pH 7.5 containing 0.1% (w/v) Triton X-100, 100 μ M DTT and 20% (v/v) ethanediol. The column was washed (2-3 column volumes) with the same buffer containing 0.1M NaCl before LMDH activity (\bullet) was eluted by applying a linear gradient (X) of 0.1 - 0.25M NaCl (total volume 2l) at a flow rate of 60ml h⁻¹ (Methods 2.24.3). Fractions were collected every 10 min and the A₂₈₀ (\blacktriangle) of the eluant was monitored.



c) Ammonium sulphate fractionation

The pooled fractions from the ion exchange column were treated with $(\text{NH}_4)_2\text{SO}_4$ (Methods 2.24.4) in order to concentrate the sample before applying to the gel filtration column. The recovery of LMDH activity in the resulting 15-30% saturation $(\text{NH}_4)_2\text{SO}_4$ fraction was very variable (25-80%). This was probably due to Triton X-100 being concentrated during the procedure causing inactivation of the enzyme. Nonetheless, a 2-3 fold purification was achieved at this stage.

d) Gel filtration chromatography

The 15-30% saturation $(\text{NH}_4)_2\text{SO}_4$ fraction was subjected to gel filtration chromatography on a column of Ultrogel AcA 34 (Methods 2.24.5). The elution profile is shown in Figure 9 and an example of a calibration curve of the column is shown in Figure 10. LMDH activity eluted at a position corresponding to an apparent M_r of 300,000. The recovery of LMDH activity from this column was approx. 60% and a 2 fold purification was achieved.

e) Monitoring the purification by SDS PAGE

The purification was monitored at this stage by SDS PAGE (Figure 11). The gel filtration pool was much purer than the ion exchange pool obtained in the preliminary experiments (Figure 7A). Although there were several contaminants in the gel filtration pool, there was a major band with an apparent M_r of 44,000 and presumably this is LMDH (see also Figure 18). The major contaminant has an apparent M_r of 66,000.

f) Testing methods for the final stage of the LMDH purification

1. Mono Q fplc column

The pooled fractions from a gel filtration column were applied to the Mono Q fplc column which had been equilibrated with 0.02M potassium phosphate buffer, pH7.5 containing 0.05% (w/v) Triton X-100, 100 μ M DTT and 10% (v/v) ethanediol. After washing the column with 2 column volumes of the same buffer, the LMDH activity was eluted by a linear gradient of 0.05M NaCl. The fractions with LMDH activity were analysed by SDS PAGE (results

Figure 9 Gel filtration chromatography of LMDH

A 15-30% saturation $(\text{NH}_4)_2\text{SO}_4$ fraction (Methods 2.24.4) was applied to a 1.41 column (71cm long x 5.2cm diam.) of Ultrogel AcA 34 which had been equilibrated with 0.02M potassium phosphate buffer, pH7.5 containing 0.05% (w/v) Triton X-100, 100 μ M DTT and 10% (v/v) ethanediol (Methods 2.24.5). LMDH activity (\bullet) was eluted by the same buffer at a flow rate of 60ml h⁻¹ and the A₂₈₀ (\blacktriangle) of the eluant was monitored.

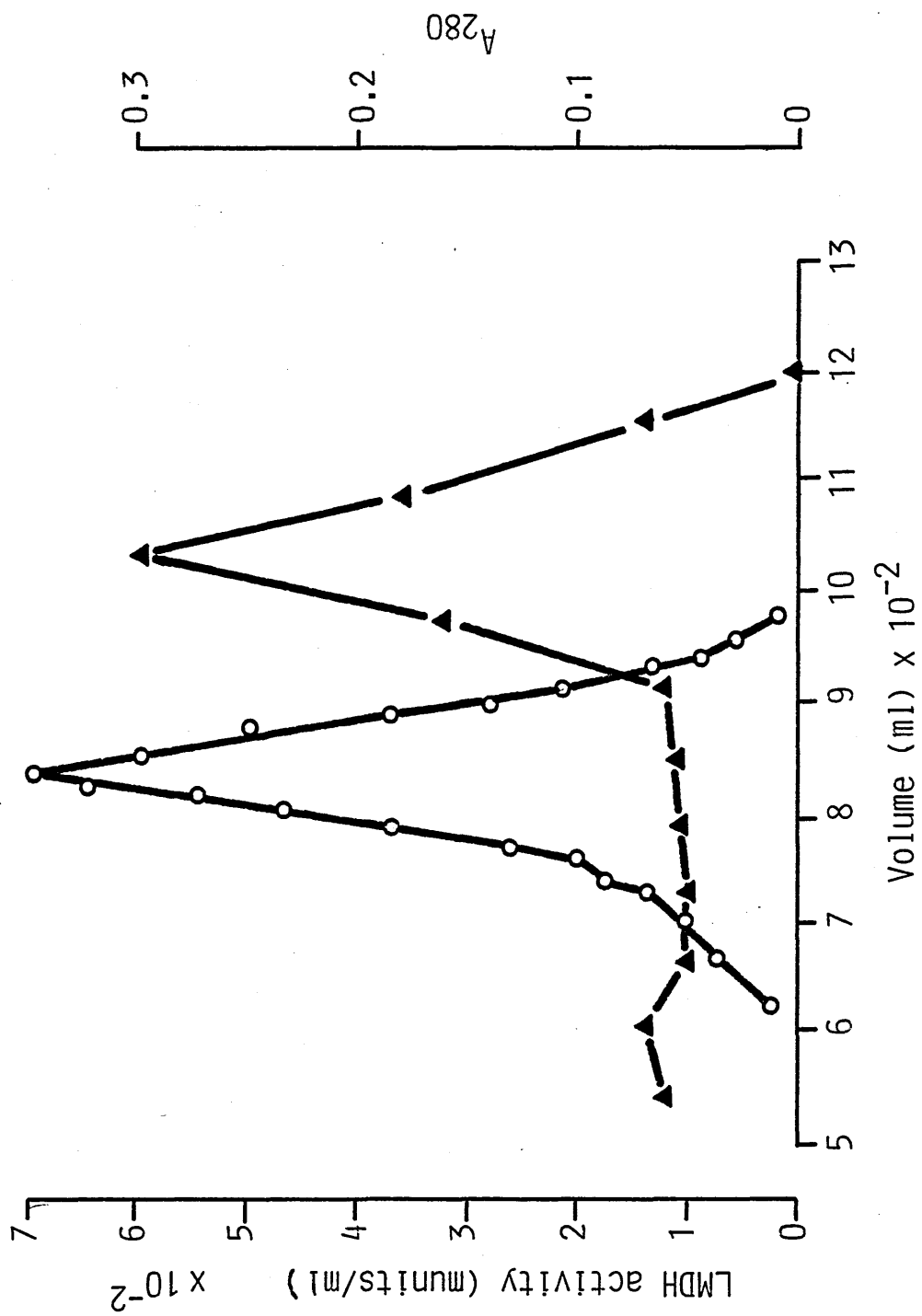


Figure 10 Calibration curve of AcA 34 (1.41) gel filtration column

The Ultrogel AcA 34 gel filtration column (71cm long x 5.2 cm diam.) was calibrated with proteins of known molecular mass in the presence (Δ) and absence (\bigcirc) of 0.05% (w/v) Triton X-100.

The proteins used were:-

	M_r
thyroglobin	669,000
ferritin	440,000
catalase	232,000
aldolase	158,000
BSA	67,000
ovalbumin	45,000
chymotrypsinogen	25,000
cytochrome c	12,300

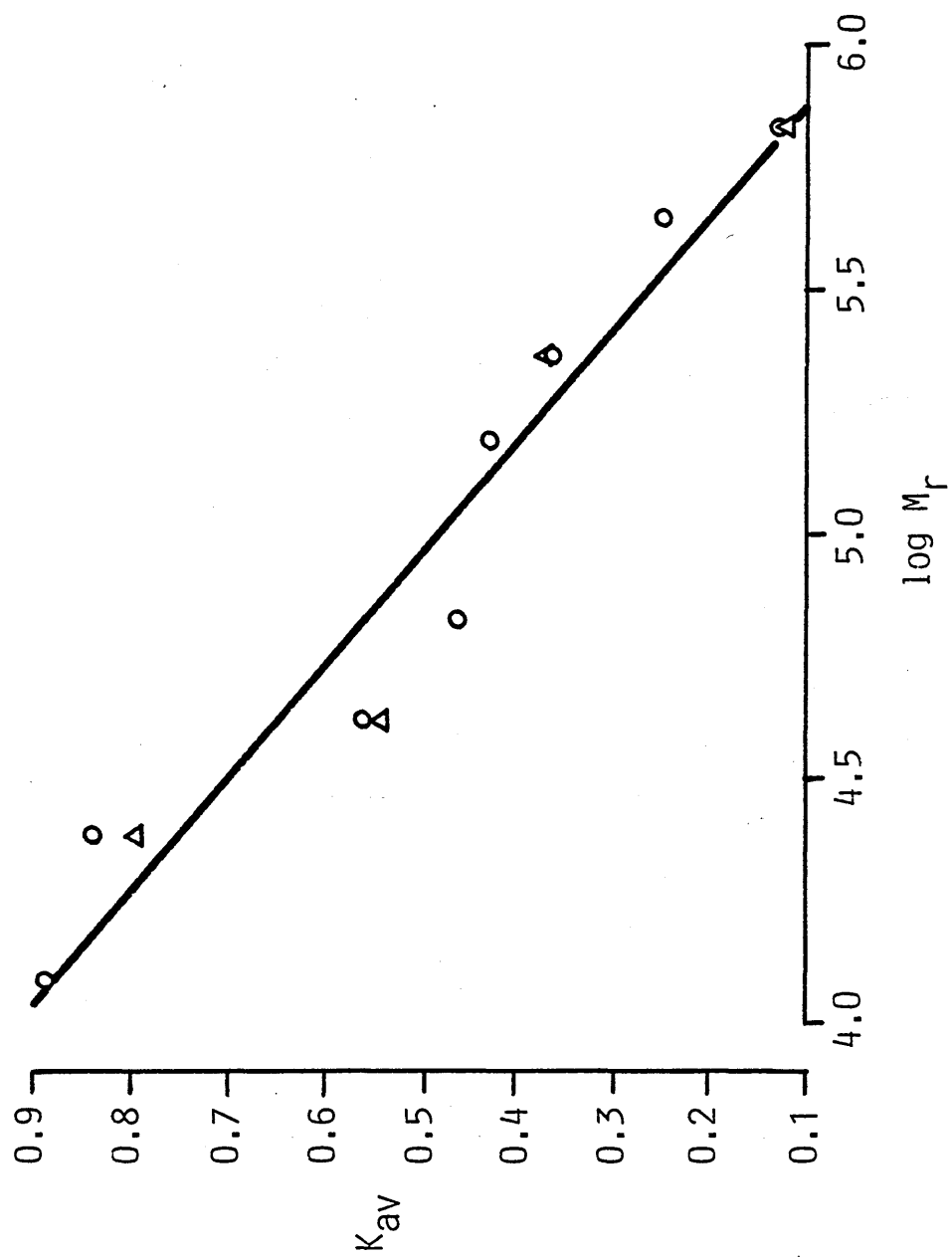
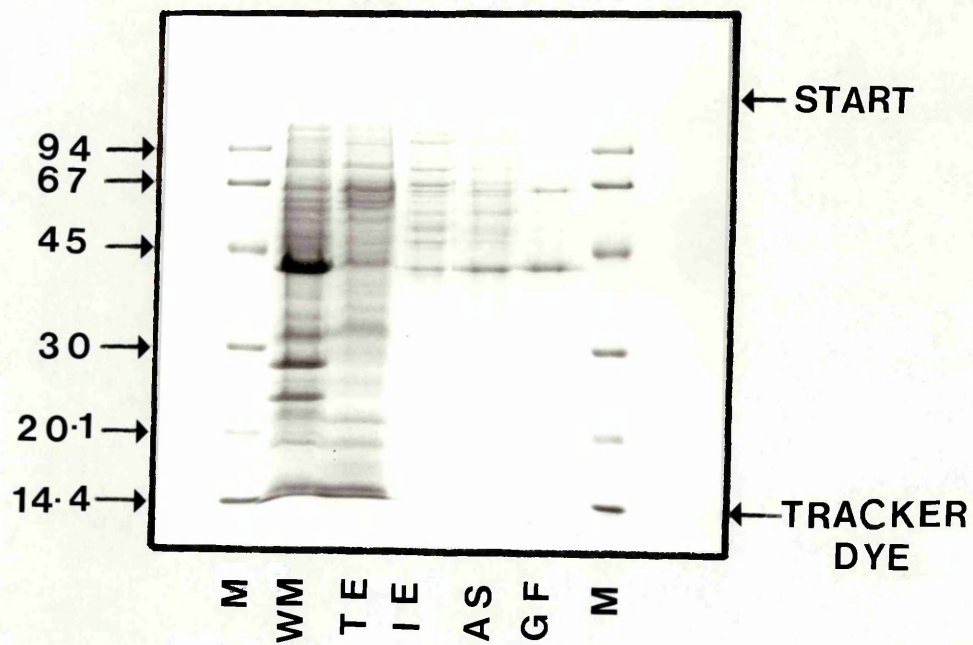


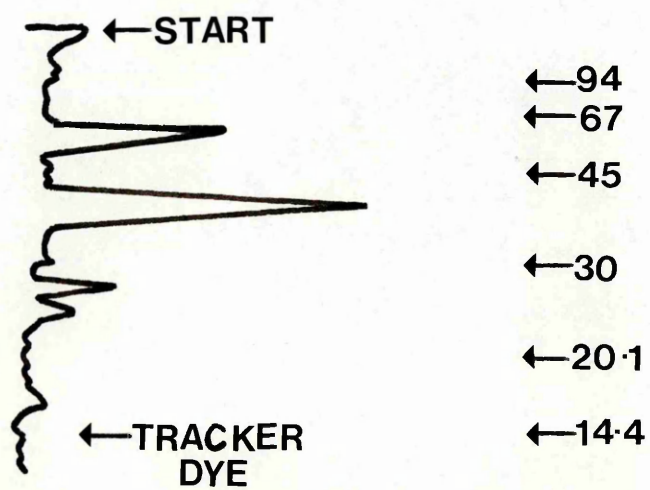
Figure 11 SDS PAGE of the LMDH purification

A sample was taken at each stage of the LMDH purification (Methods 2.24) and subjected to SDS PAGE (Methods 2.21.4) on a 12.5% gel which was stained with Coomassie Blue (A). Approx. 50 μ g of protein (or 10 μ g in the case of the gel filtration pool) was loaded per track. The gel was calibrated with proteins of known molecular mass (M) from Pharmacia:- phosphorylase b, M_r 94,000; bovine serum albumin, M_r 67,000; ovalbumin, M_r 45,000, carbonic anhydrase, M_r 30,000; soyabean trypsin inhibitor, M_r 20,100 and α -lactalbumin, M_r 14,400. WM = 'wall + membrane' fraction; TE = Triton X-100 extract; IE = ion exchange pool; AS = 15-30% saturation ammonium sulphate fraction, GF = gel filtration pool which had been concentrated 10 fold (Methods 2.26.1). The gel scan of the LMDH gel filtration pool is shown (B).

A

 $M_r \times 10^{-3}$ 

B

 $M_r \times 10^{-3}$ 

not shown) and the contaminating band with an apparent M_r of 66,000 was present in all fractions across the peak. Varying the gradient and changing the pH to 6.5 did not allow purification of LMDH.

2. Adsorption chromatography using hydroxyapatite (HA) Ultrogel

At the same time as these experiments on LMDH purification were carried out, Dr. N. Allison was working on the LLDH purification. This enzyme had been purified to 80% homogeneity using HA Ultrogel at the final stage of the purification. Preliminary experiments with LMDH on a column of HA Ultrogel were carried out by Mr. A. Scott. Better recovery of activity was obtained when the chromatography was run at pH6.5 and so in these experiments the gel filtration column was equilibrated and run in 0.05M MES buffer, pH6.5 containing 0.05% (w/v) Triton X-100, 100 μ M DTT and 10% (v/v) ethanediol. The LMDH gel filtration pool was applied to a column of HA Ultrogel equilibrated with the same buffer and LMDH activity was eluted from the column by a linear gradient of 0-100mM KH_2PO_4 . When the fractions were analysed by SDS PAGE (results not shown) it was seen that although the contaminating proteins eluted at a lower KH_2PO_4 concentration (15mM) than the LMDH activity (30mM), the major band was also present in the fractions containing LMDH activity (due to trailing). Attempts to manipulate the gradient so that the contaminants could be washed from the column (by washing with 15mM KH_2PO_4) before eluting the LMDH activity were unsuccessful. This was because when the column was washed with buffer containing 15mM KH_2PO_4 , both the contaminating proteins and the LMDH eluted at higher KH_2PO_4 concentrations (75mM and 90mM respectively) than previously observed. In addition, there were two peaks of LMDH activity. This irreproducible behaviour on the HA Ultrogel may indicate that more than one mechanism is involved in separation on this column e.g. the HA Ultrogel may also be behaving as an ion exchanger.

3. Ion exchange chromatography II

An LMDH gel filtration pool was subjected to ion exchange chromatography on a column of DEAE Sephacel (Figure 12). There was good recovery (60%) of LMDH activity from this column, although, in an effort to obtain a purer sample, only 40% of the activity was pooled. The pool containing LMDH activity was concentrated (Methods 2.26.1) and subjected to SDS PAGE (Figure 13). Since there was very little protein in this final sample, concentration by ammonium sulphate precipitation resulted in a substantial loss of protein owing to the difficulty in recovering the floating material (see Methods 2.24.4). Alternative methods of concentrating the sample e.g. using MiniconTM_B macrosolute concentrators (Amicon Corporation, Danvers, M.A. 01923, USA) were also unsatisfactory possibly because Triton-protein micelles adhered to the membranes of these concentrators. As much as possible of the concentrated ion exchange II LMDH pool was applied to the SDS polyacrylamide gel shown in Figure 13A and it can be seen from the gel scan (Figure 13B) that there was some contamination of the LMDH. The procedure outlined in Methods 2.24 resulted in the purification of LMDH almost to homogeneity and the purification is summarised in Table 3.

Figure 12 Ion exchange chromatography II of LMDH

A gel filtration pool of LMDH (Methods 2.24.5) was applied to a column (5.0cm long x 2.6cm diam.) of DEAE Sephacel which had been equilibrated with 0.02M potassium phosphate buffer, pH7.5 containing 0.05% (w/v) Triton X-100, 100 μ M DTT and 10% (v/v) ethanediol. The column was washed with 2-3 column volumes of the same buffer containing 0.1M NaCl before LMDH activity (\circ) was eluted by applying a linear gradient (X) of 0.1 - 0.25M NaCl (total volume 500ml) at a flow rate of 20ml h⁻¹ (Methods 2.24.6). The A₂₈₀ (\blacktriangle) of the eluant was monitored.

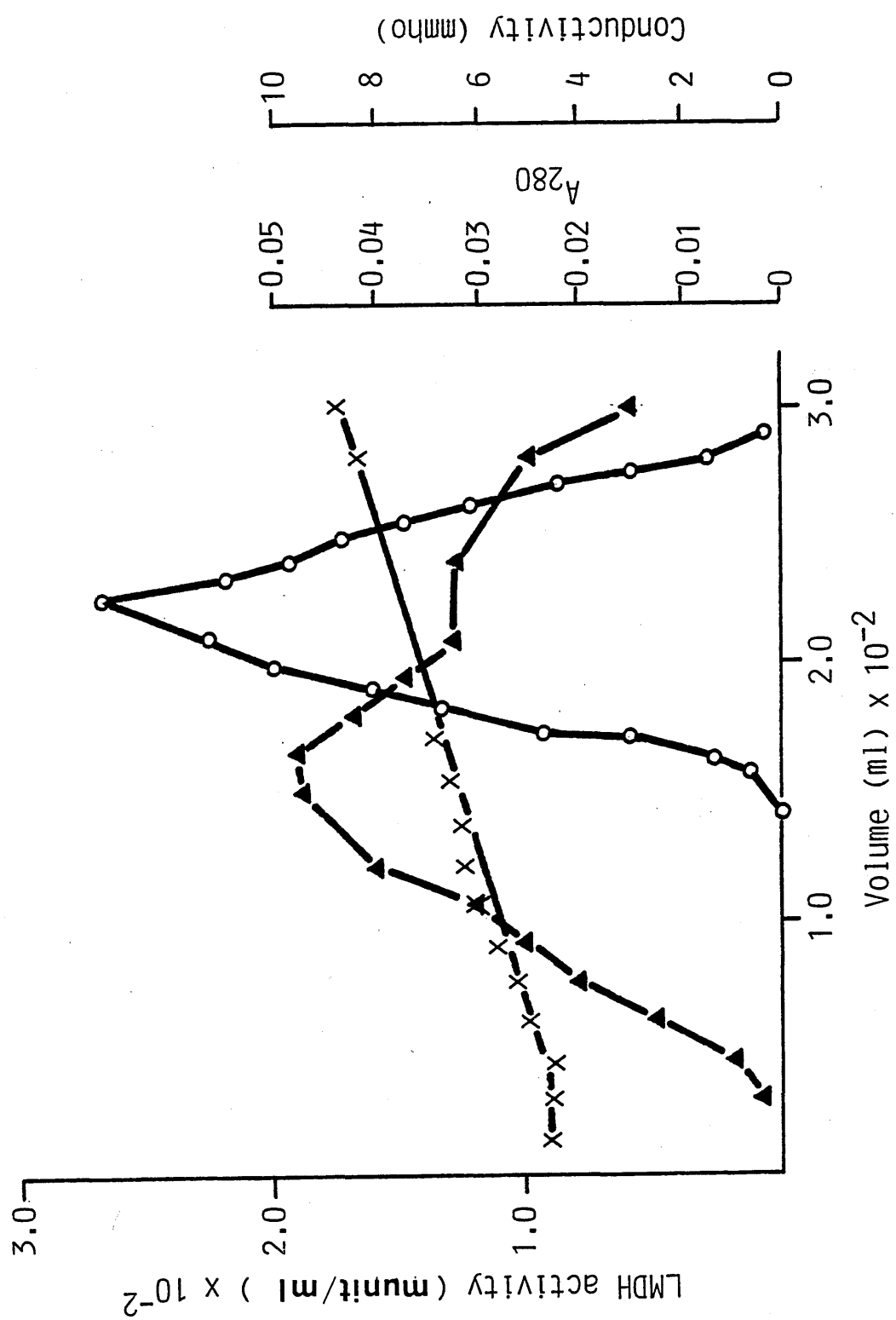


Figure 13 SDS PAGE of the gel filtration and ion exchange II LMDH pools

The pools from a gel filtration (Methods 2.24.5) and ion exchange II column (Methods 2.24.6) were subjected to SDS PAGE (Methods 2.21.4) on a 12.5% gel which was calibrated with Pharmacia molecular mass proteins (M).

- A. Coomassie Blue stained gel; GF = gel filtration LMDH pool;
IE II = ion exchange II LMDH pool. Both samples were concentrated (Methods 2.24.4) 10 fold before applying (approx. 10 μ g and 4 μ g protein respectively) to the gel.
- B. Gel scan of the ion exchange II LMDH sample.

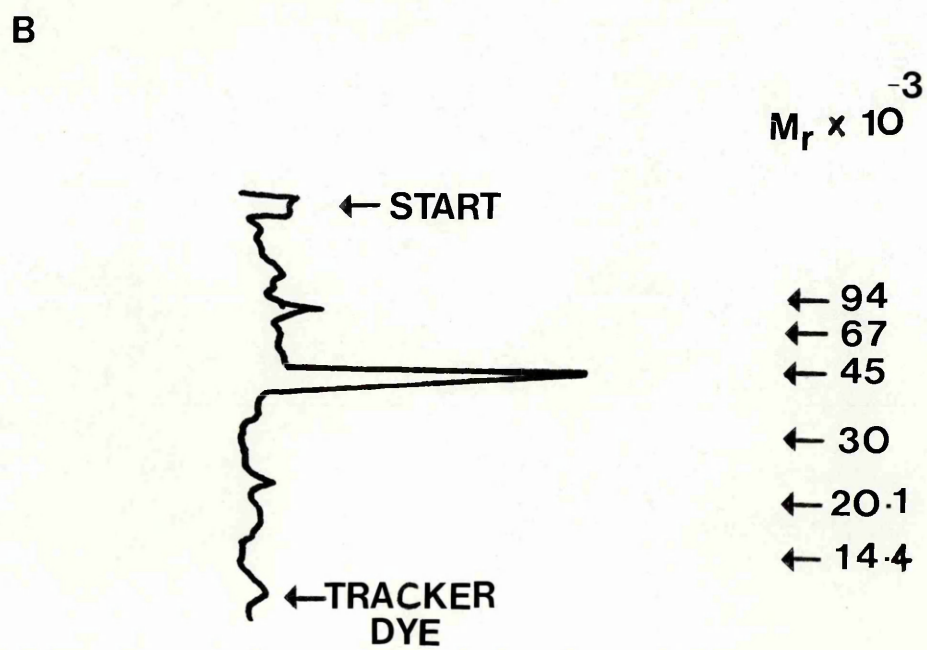
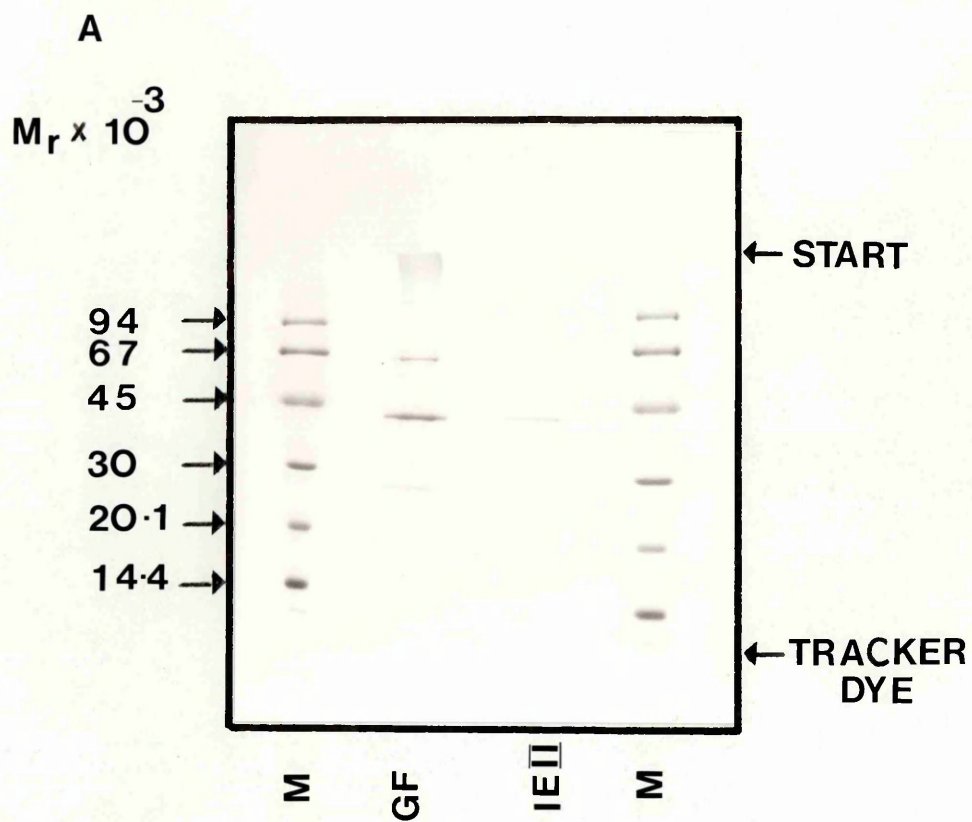


Table 3

Purification of LMDH from A.calcoaceticus mutant strain C1005

	Volume (ml)	Protein concentration (mg ml ⁻¹)	Total activity (units)	Specific activity (units/mg protein)	Purification factor	Yield (%)
'Wall + membrane' fraction	180	20.0	1502	0.417	1	100
Triton extract	208	3.23	1154	1.72	4.12	76.8
Ion exchange I	194	0.25	283	5.84	14.0	18.8
(NH ₄) ₂ SO ₄ 15-30% saturation	31	0.231	75	10.47	25.1	5.0
Gel filtration	136	0.034	66	14.21	34.1	4.4
Ion exchange II	103	0.01	17	16.12	38.7	1.1

LMDH was purified by the method outlined in Methods 2.24

Section 3.3

Characterisation of purified L-mandelate dehydrogenase

3.3.1 Introduction

The properties of the purified LMDH were studied to allow a comparison with those of the purified DMDH, DLDH and LLDH.

3.3.2 Relative molecular mass

Analysis of the purified LMDH by SDS PAGE gave an apparent M_r of $44,000 \pm 800$ ($n=20$). Gel filtration of the purified sample on a column (30cm long x 0.7cm diam.) of Ultrogel AcA 34 which had been calibrated with proteins of known molecular mass gave an apparent M_r of 64,000 (average of two determinations).

3.3.3 The effect of pH on LMDH activity

The enzyme has a fairly broad pH range with the half maximal activity at pH5.2 and pH8.95 (Figure 14). The optimum pH of the purified LMDH is pH7.5.

3.3.4 Determination of K_m

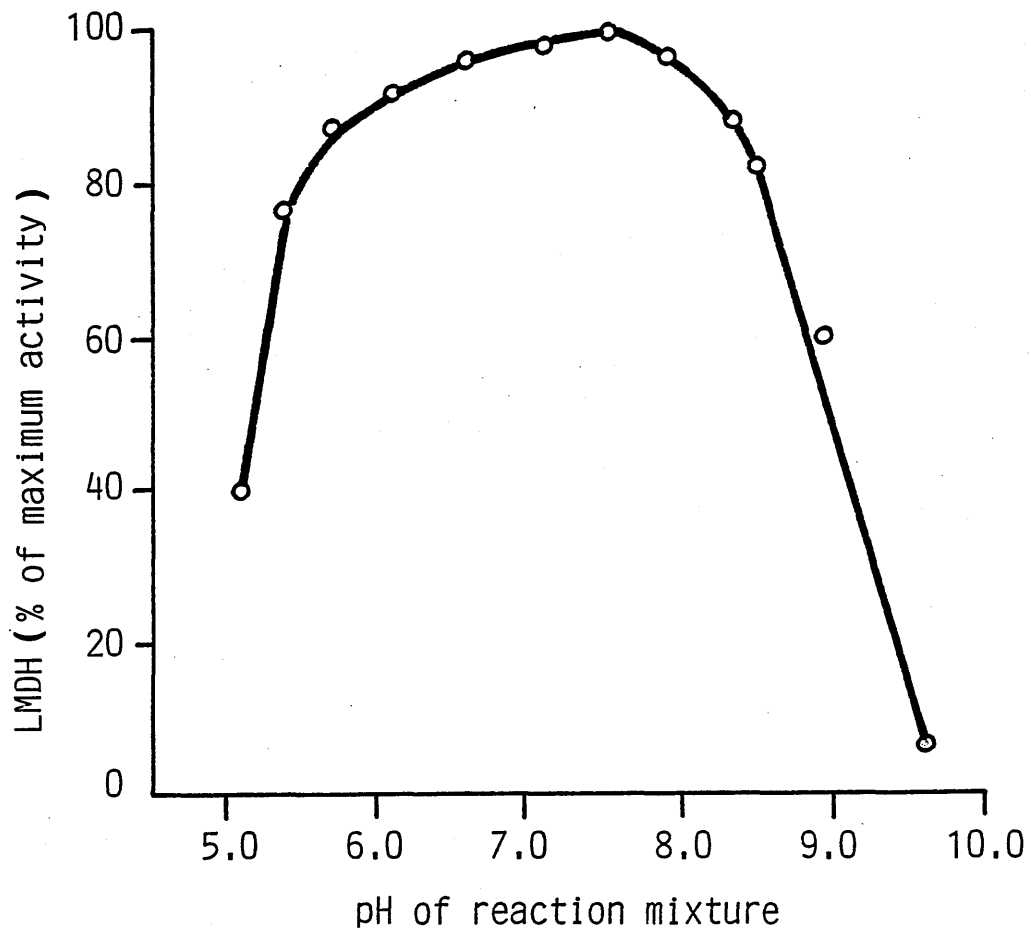
The K_m of the purified LMDH was calculated by measuring the activity at different substrate concentrations (Methods 2.30). For convenience the results are illustrated as direct plots and as Lineweaver-Burk plots (Figure 15). Using the Direct Linear plot on the Enzpack computer programme (Williams, 1985), the calculated K_m was $186 \pm 19\mu\text{M}$ ($n=3$).

3.3.5 The effect of substrates on LMDH activity

LMDH was found to be completely stereospecific for its substrate i.e. L-mandelate but not D-mandelate was oxidised by this enzyme. Neither D-lactate nor L-lactate gave detectable rates of DCIP reduction even at a final concentration of 2mM.

D-mandelate inhibited LMDH activity only at high concentrations ($\geq 10\text{mM}$) and only when L-mandelate was present at a low concentration e.g. when L-mandelate was present at a concentration of $100\mu\text{M}$, 10mM D-mandelate inhibited LMDH by 36% and 20mM D-mandelate inhibited LMDH by 60%. L-lactate and D-lactate did not affect LMDH activity even at

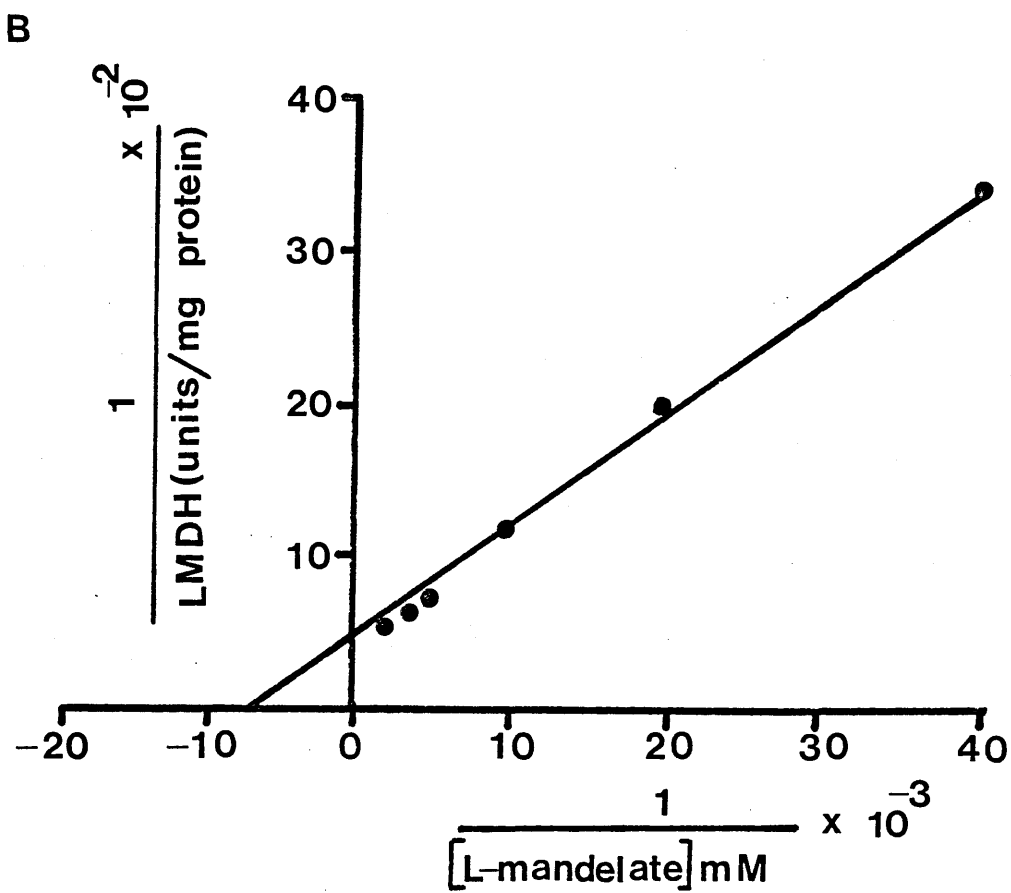
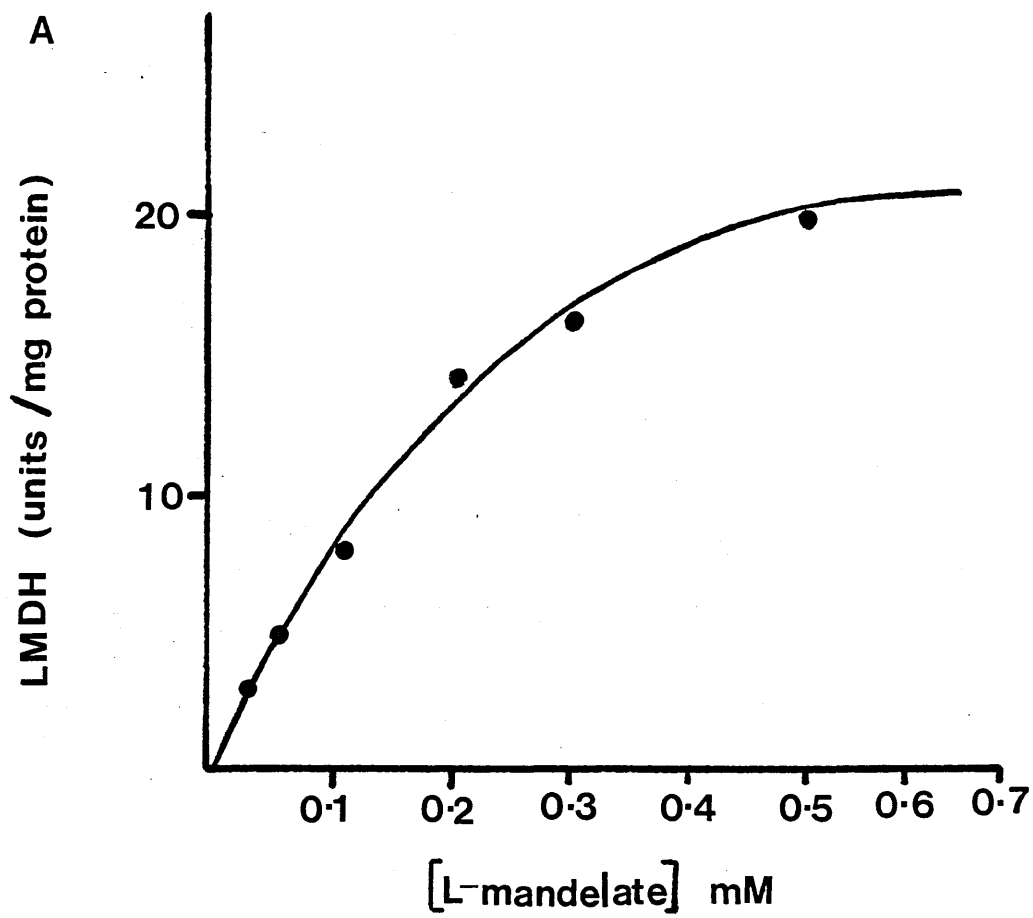
Figure 14

The effect of pH on LMDH activity

The pH of the LMDH assay was varied over the range pH5.1 to pH9.6 by adjusting the pH of the phosphate buffer. The absorption coefficient for DCIP was determined at each pH and used in the calculation of rates. The percentage of the maximum activity is plotted against the actual pH of the reaction mixture.

Figure 15 Determination of K_m of purified LMDH

An example of the results obtained when LMDH activity was determined at six different substrate concentrations is plotted as a direct plot (A) and as a Lineweaver-Burk plot (B) which has a correlation coefficient of 0.997.



concentrations of 20mM in the presence of 100 μ M L-mandelate.

3.3.6 The effect on LMDH activity of reagents which react with sulphydryl groups

The effect on LMDH activity of various thiol group inhibitors is shown in Table 4. Iodoacetate had no effect on the enzyme activity. The enzyme was slightly more sensitive to inhibition by N-ethylmaleimide than iodoacetamide. LMDH was completely inhibited by 10 μ M mercuric chloride whereas 30 μ M p-chloromercuribenzoate inhibited the enzyme by 53%.

3.3.7 The effect on LMDH activity of metal chelating reagents and related compounds

LMDH was unaffected by the metal chelators EDTA and 2,2'-bipyridyl at the concentrations tested (Table 5). Similarly, azide and cyanide did not effect enzyme activity.

3.3.8 Analysis of flavin

The difference spectrum of pure LMDH with and without substrate (Figure 16) showed a peak at 400nm with a shoulder between 420 and 460nm. Addition of dithionite produced almost exactly the same effect as substrate (Figure 16).

Treatment of the purified LMDH with cold TCA (Methods 2.25) resulted in an extract whose emission fluorescence spectrum (exciting at 420nm) was identical to that of standard solutions of FAD and FMN (Figure 17). The emission fluorescence spectrum of the standard solutions and of the TCA extract of LMDH was abolished by the addition of dithionite. The addition of a small crystal of Naja naja snake venom to the standard solution of FAD resulted in a 9 fold increase in the fluorescence at 520nm (Figure 17A) corresponding to the conversion of FAD to FMN by the phosphodiesterase in the snake venom (Bessey et al., 1949). However, the Naja naja snake venom had no effect on the emission fluorescence spectrum of the standard solution of FMN or of the TCA extract of LMDH (Figure 17B,C). This suggests that FMN is the flavin prosthetic group

Table 4 The effect on LMDH activity of reagents which react with sulphhydryl groups

Inhibitor	Concentration (mM)	% activity remaining
N-ethylmaleimide	1	64
	5	23
Iodoacetamide	5	66
	10	31
Iodoacetate	5	101
	10	98
Mercuric chloride	0.001	50
	0.01	0
p-Chloromercuribenzoate	0.03	47
	0.3	24

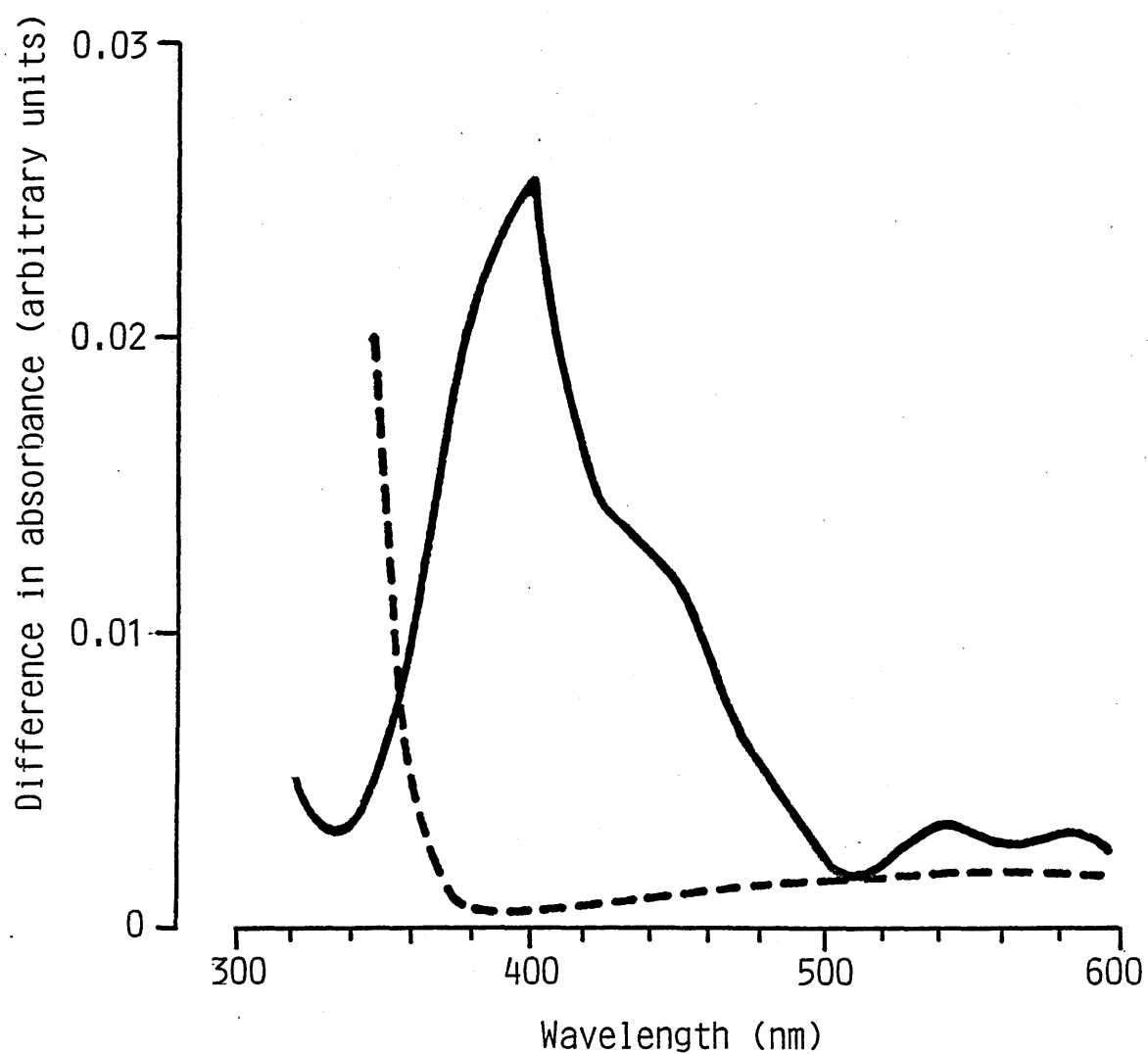
The inhibitors were preincubated with the enzyme and phosphate buffer (total volume 2.5ml) for 10 min at 27°C before addition of the remaining reaction components. The inhibitor concentration refers to the final concentration in the assay.

Table 5 The effect on LMDH activity of metal chelators and related components

Potential inhibitor	Concentration (mM)	% activity remaining
EDTA	1	98
2,2'-bipyridyl	4	97
KCN	10	102
NaN ₃	10	101

The potential inhibitors were preincubated with the enzyme and phosphate buffer (total volume 2.5ml) for 10 min at 27°C before the addition of the remaining reaction components. The concentration refers to the final concentration in the assay.

Figure 16 Difference spectrum of purified LMDH

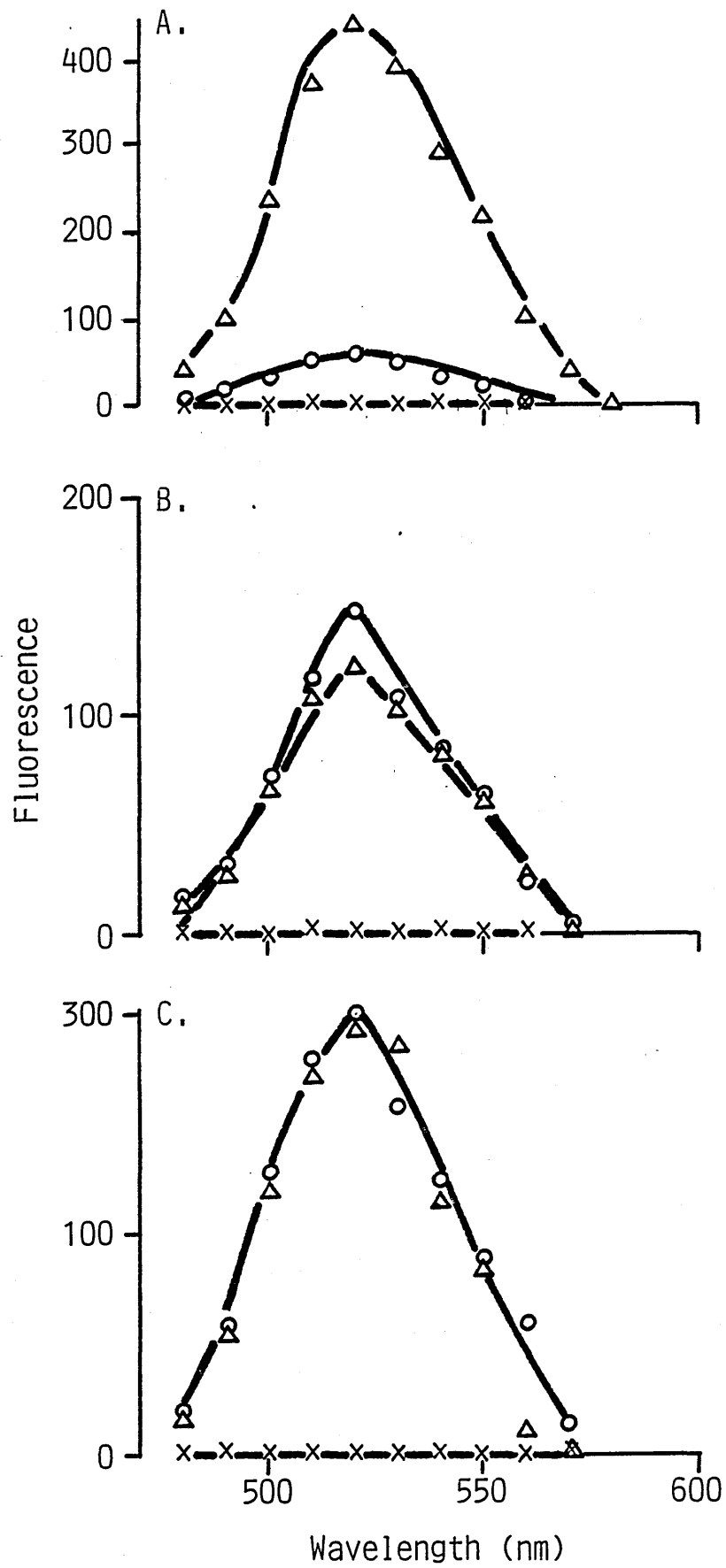


The difference spectrum of the purified enzyme was obtained by measuring absorption spectra thus -

Reference cell	Sample cell
LMDH + 6 μ mol L-mandelate	LMDH —————
LMDH + 6 μ mol L-mandelate	LMDH + dithionite - - - - -

Figure 17 The emission fluorescence spectra of standard solutions of FAD and FMN and of a TCA extract of LMDH

The emission fluorescence spectra, exciting at 420nm (Methods 2.25) of a standard solution (0.25 μ M) of FAD (A) and of a standard solution (1 μ M) of FMN (B) and of a TCA extract of LMDH (C) is shown (○). The effects of the addition of dithionite (X) and of Naja naja snake venom (Δ) are shown.



of LMDH.

3.3.9 Preparation of antiserum against LMDH

The recovery of LMDH at the final stage of the purification was poor and so a less purified sample (a concentrated gel filtration pool) was used to prepare the antigen (Methods 2.26). The sample was run on a 12.5% SDS polyacrylamide preparative gel (Methods 2.26.2; Figure 18) and the band corresponding to LMDH was cut out and treated as described in Methods 2.26.2.

3.3.10 Antibody - enzyme inhibition studies

Studies to investigate possible enzyme inhibition by the antiserum raised against LMDH were carried out on sonic extracts of mutant strain Cl219 and of mutant strain Cl005 and on the purified enzyme (Methods 2.27). However, under conditions which resulted in 73% inhibition of DMDH by anti-DMDH and 95% inhibition of PGDC by anti-PGDC, no inhibition of LMDH was observed in crude extracts or of the purified enzyme by the antiserum raised against LMDH.

3.3.11 Immunoblots of extracts from various *A. calcoaceticus* strains with anti-LMDH

Extracts from non-induced strain NCIB 8250 (no LMDH activity present), induced strain NCIB 8250 (LMDH activity present), mutant strain Cl005 (LMDH activity present), mutant strain Cl219 (LMDH activity present), mutant strain Cl123 (no LMDH activity present) and mutant strain Cl408 (no LMDH activity present) were blotted with antiserum raised against LMDH (Figure 19A).

There was a band with an M_r value of approx. 44,000 in extracts of induced NCIB 8250, mutant strain Cl005 and mutant strain Cl219 i.e. those extracts with LMDH activity but not in the other extracts which had no LMDH activity. In addition there was a major contaminating band ($M_r = 61,000$) in all extracts as well as a few other minor contaminants.

Figure 18 IMDH preparative gel used to prepare the IMDH antigen

A concentrated gel filtration pool of IMDH (Methods 2.26.1) containing 250 μ g of protein in 1.5ml was run on a 12.5% preparative gel (Methods 2.26.2) which was stained with Coomassie Blue to reveal the band pattern shown. The band corresponding to IMDH was cut out of the gel and ground in liquid N₂. The resulting powder was prepared for injection (Methods 2.26.2).

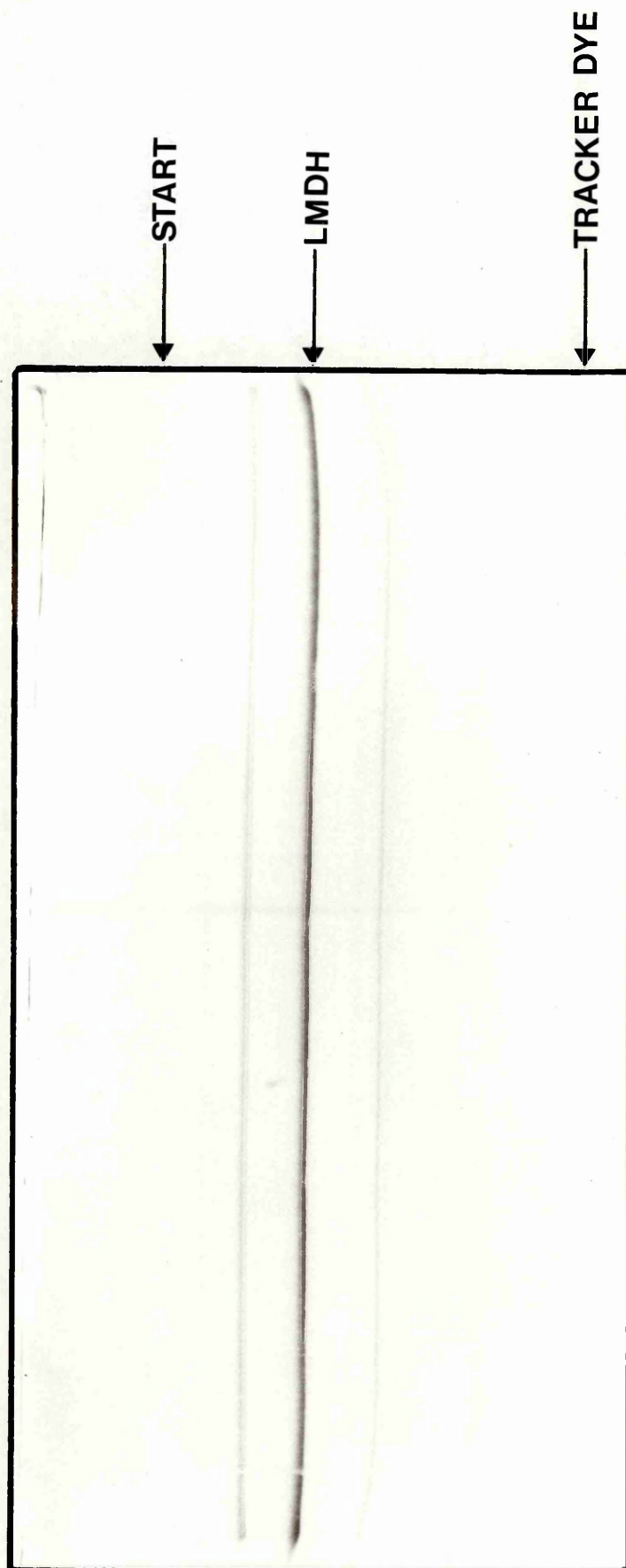
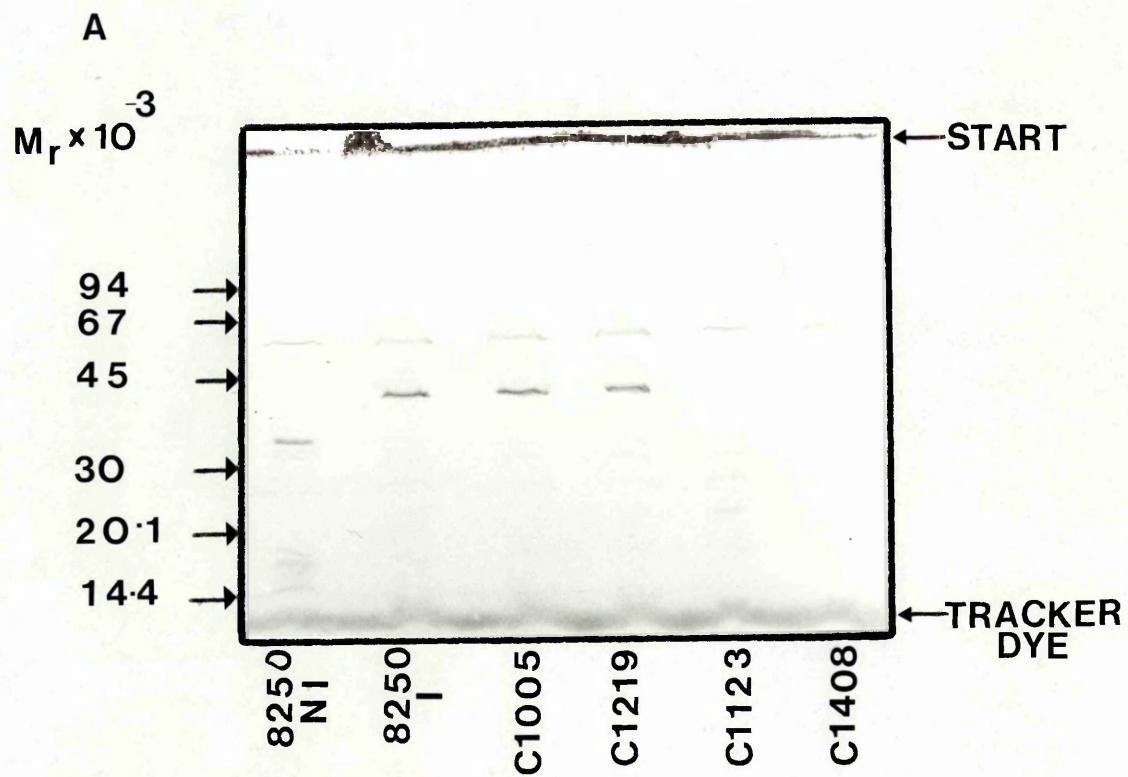


Figure 19 Immunoblots of sonic extracts from various strains of
A.calcoaceticus with anti-LMDH

Sonic extracts from strain 8250 grown on succinate (Methods 2.7.3) and from strains 8250, C1005, C1219 and C1123 grown on complex media supplemented with D,L-mandelate and from mutant strain C1408 grown on complex media supplemented with phenylglyoxylate were subjected to SDS PAGE on 12.5% gels (Methods 2.21.4) and subsequent immunoblotting with anti-LMDH (Methods 2.22) before (A) and after (B) adsorption of the anti-LMDH with non-induced extract (Methods 2.26.4). NI = non-induced; I = induced. The positions of standard molecular mass proteins (Pharmacia) are indicated. These were run alongside the extracts but were stained for protein (not shown).



Since the contaminating bands were present in the blot of the non-induced 8250 extract it was hoped that the anti-LMDH could be adsorbed using this extract. In initial experiments, anti-LMDH was incubated with a non-induced extract from NCIB 8250 grown on succinate (Methods 2.7.3); however, there was no change in the immunoblotting pattern observed after this treatment. When the non-induced extract was first denatured and then incubated with anti-LMDH (Methods 2.26.4), only one band, corresponding to LMDH, was detected on immunoblots of extracts from induced NCIB 8250, mutant strain C1005 and mutant strain C1219 (Figure 19B). So, although the antiserum raised against LMDH does not inhibit activity (Results 3.3.10), it gives a positive reaction on immunoblotting extracts with LMDH activity.

Section 3.4

Development of PGDC assay

3.4 Development of PGDC assay

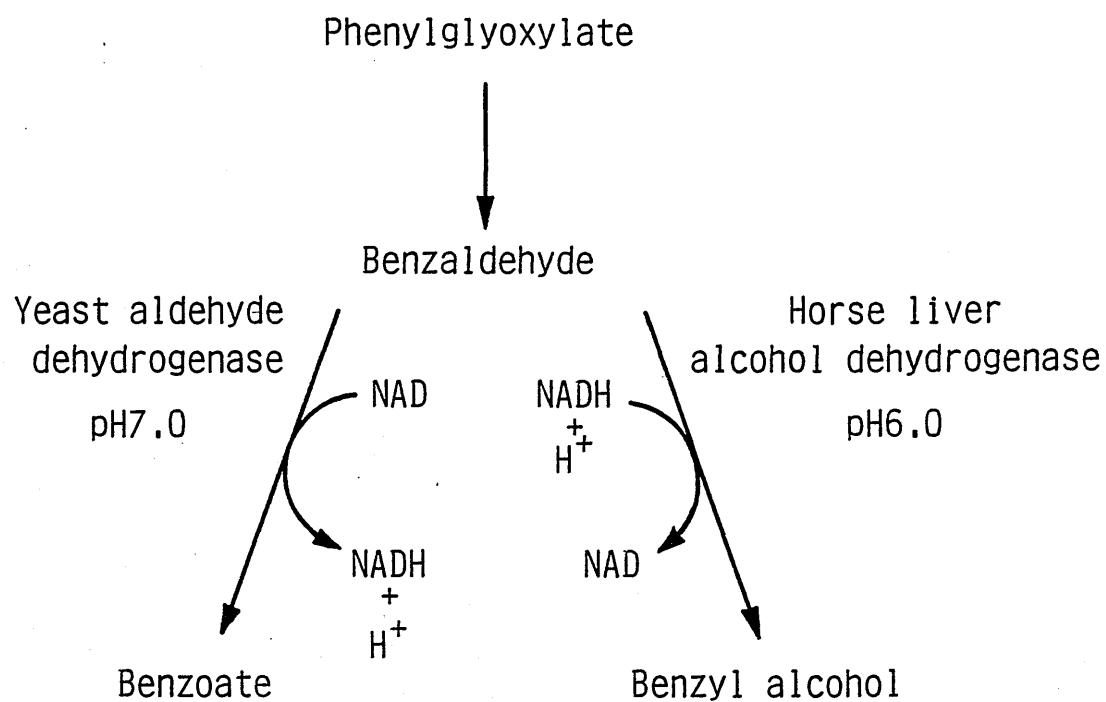
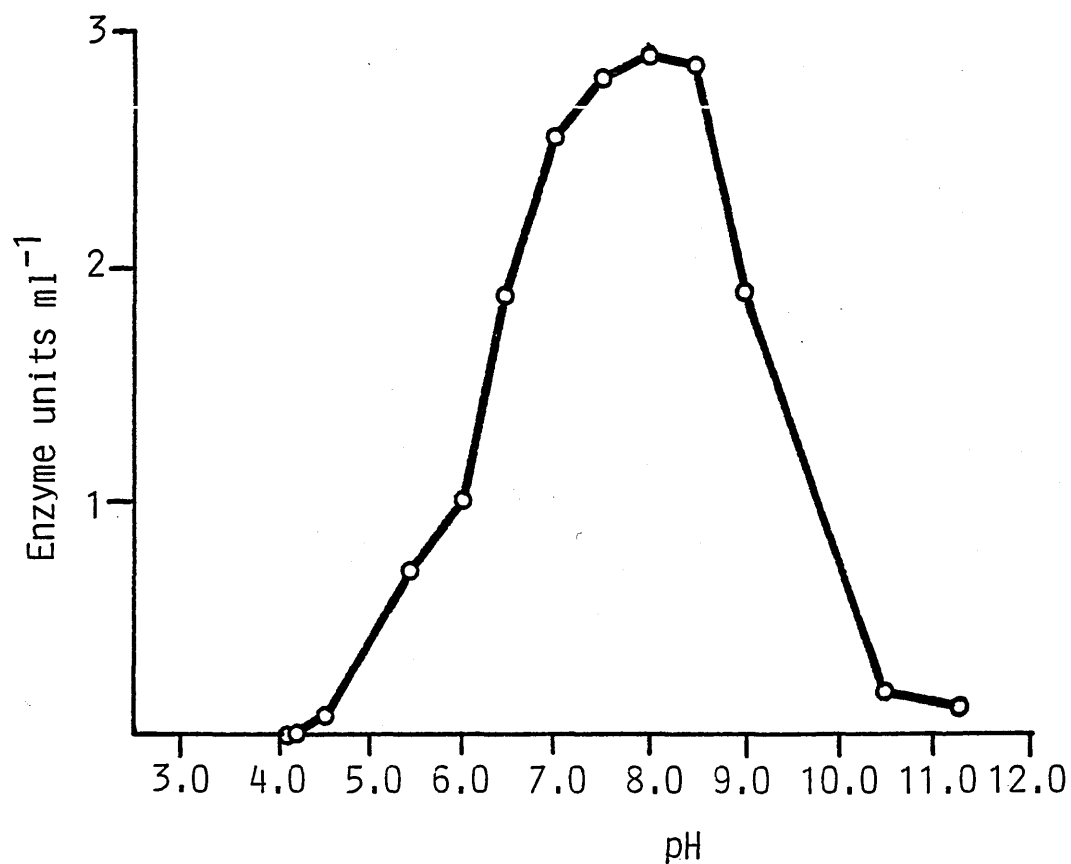
During the course of this study, problems were encountered with the PGDC assay procedure. In some cases the correlation between results using the direct PGDC assay (Methods 2.17.3) and the yeast aldehyde dehydrogenase-linked PGDC assay (Methods 2.17.4) was very poor. When NADH oxidase activity was present in the samples, the PGDC value obtained with the aldehyde dehydrogenase-linked assay was much lower than that obtained with the direct PGDC assay. This was probably a result of NADH oxidase consuming the NADH produced during the coupled aldehyde dehydrogenase reaction (Figure 20A).

An alternative assay was therefore developed involving coupling PGDC activity with horse liver alcohol dehydrogenase by a modification of the method described by Ullrich (1970) for pyruvate decarboxylase. The use of horse liver alcohol dehydrogenase in a coupled assay has several advantages:

- a) the coupled reaction involves the oxidation of NADH (Figure 20A) and it should therefore be possible to correct for NADH oxidase activity in samples by including a minus substrate (phenylglyoxylate) cuvette as a control.
- b) this coupled reaction is measured at pH6.0 (the optimum pH for PGDC) at which pH the NADH oxidase activity is decreased (Figure 20B).
- c) the equilibrium favours benzyl alcohol formation, especially at low pH.

Since potassium phosphate is a poor buffer at pH6.0, PGDC activity was measured in potassium phosphate buffer, pH6.0 and in MES buffer, pH6.0; nevertheless a higher PGDC activity was obtained in the presence of potassium phosphate buffer.

The concentrations of potassium phosphate buffer, NADH, TPP, phenylglyoxylate and alcohol dehydrogenase were varied systematically and the assay condition described in Methods 2.17.5 resulted in maximal PGDC

A. Mechanism of indirect PGDC assays.B. pH profile of NADH oxidase

activity.

For each set of samples, the assays were carried out with 0.5, 1.0 and 2.0 times the amount of alcohol dehydrogenase in the assay to ensure that the alcohol dehydrogenase was present in non-rate limiting concentrations. In most cases, this was only true when the extract to be assayed was diluted. When alcohol dehydrogenase was present in non-rate limiting concentrations, the assay rate was proportional to the amount of enzyme extract added and the assay was linear with respect to time upto approx. 10 min.

The reproducibility of the alcohol dehydrogenase-linked PGDC assay was studied by assaying different volumes of a given sample. For example in an experiment using a sonic extract of mutant strain C1005 grown on complex media supplemented with D,L-mandelate, a value for PGDC activity of 926 ± 26 enzyme units ml^{-1} ($n=6$) was obtained by the alcohol dehydrogenase-coupled assay. In addition, the same sample was assayed by the direct PGDC assay procedure (Methods 2.17.3) and a value of 976 ± 31 enzyme units ml^{-1} ($n=6$) was obtained, illustrating a good correlation between the two assay procedures.

3.5.1 Crosslinking intact Pseudomonas putida with DMSI

Halpin et al. (1981) claimed to have evidence for an association between the mandelate enzymes of P.putida and so as a first step, some of the experiments described in this paper were repeated and expanded.

P.putida were treated with DMSI in the presence of TNM buffer, pH8.0 as described by Halpin et al. (1981) and in Methods 2.14.3. In addition, aldolase was treated with DMSI at the same time but separately to check that the crosslinking reagent was working. After disrupting the bacteria by sonication the extracts were analysed by high speed centrifugation as specified by Halpin et al. (1981). Table 6 shows the results obtained from control and DMSI treated P.putida. In these experiments catechol-1, 2-dioxygenase (CO) was used as a control 'soluble' enzyme from a separate but related part of metabolism (Figure 1) and isocitrate dehydrogenase (ICDH) and glutamine synthetase (GS) were used as control 'soluble' enzymes from unrelated parts of metabolism. Furthermore, GS served as an example of an enzyme with a high M_r (i.e. 600,000) whereas ICDH was an example of an enzyme with a lower M_r (i.e. 100,000). Halpin et al. (1981) also used GS as a control enzyme but they assayed cis, cis-muconate lactonising enzyme (Figure 1) instead of CO.

In general, DMSI did not affect the total specific activities of the enzymes, although LMDH was slightly inhibited (Table 6). There was a 5-50 fold increase in the specific activities of the 'soluble' enzymes of the mandelate pathway present in the high speed pellet after DMSI treatment. However, this was not a specific effect since the specific activities of CO, ICDH and GS in the pellet were also increased by the same extent (Table 6). No increase in the specific activities of the enzymes in the pellet was observed after DMSI treatment of stationary phase bacteria (results not shown).

The results reported by Halpin et al. (1981) are shown in Table 7 and it can be seen that these data are similar to those shown in Table 6.

Table 6 Crosslinking intact *P.putida* with DMSI

Enzyme	Total specific activity (munits/mg protein)		Specific activity in pellet (munits/mg protein)		% recovered in pellet	
	Control	DMSI	Control	DMSI	Control	DMSI
LMDH	309	247	661	622	95	97
Mandelate- racemase	433	408	2.2	100	0.2	9.4
PGDC	215	248	2.9	13.3	0.6	2.1
NAD-BDH	79	76	ND	ND	ND	ND
NADP-BDH	49	56	0.8	7.3	0.7	5
CO	498	499	2.7	40	0.2	3.1
ICDH	524	412	2.9	33	0.3	3.1
GS	25	33	2.3	18	4.2	22
Protein	-	-	-	-	45	38

P.putida was grown on minimal mandelate media (Methods 2.7.4), harvested in late exponential phase and treated with DMSI in the presence of TNM buffer, pH8.0 (Methods 2.14.3). After disrupting the bacteria by sonication (Methods 2.9.1), the extracts were centrifuged at 105,000g_{av} (40K rpm, Ti50 rotor) for 60 min and the 'high speed pellets' so formed were washed three times by resuspension/centrifugation. The pellets, supernatants and washes were assayed for enzyme activity so that the results could be expressed in terms of the percentage of the total enzyme activity recovered. The total specific activities (here and in all subsequent experiments) were derived from the addition of the enzyme units and protein recovered in the pellet, supernatant and washes of each sample. ND = not detectable.

Table 7 Results of Halpin et al. (1981) for the crosslinking of intact P.putida with DMSI

Enzyme	Specific activity in pellet (munits/mg protein)	
	Control	DMSI .
LMDH	746	629
Mandelate-racemase	8	178
PGDC	2	18
<u>cis,cis</u> -muconate- lactonising enzyme	2	23
GS	0	32

The pellet was obtained from sonic extracts from control and DMSI treated bacteria by centrifuging at 100,000g for 60 min.

An 11-32 fold increase in the specific activities of the control enzymes (cis, cis-muconate lactonising enzyme and GS) was reported but no emphasis was placed on this (Halpin et al., 1981).

Halpin et al. did not express their results in terms of total percent of enzyme, nor did they provide sufficient information in this paper so that the percentages could be calculated. When the results from my experiments were expressed in this way (Table 6) it can be seen that the amount of the 'soluble' mandelate enzymes recovered in the pellet fraction after DMSI treatment (2-9%) is very small in relation to the amount of LMDH (97%) present.

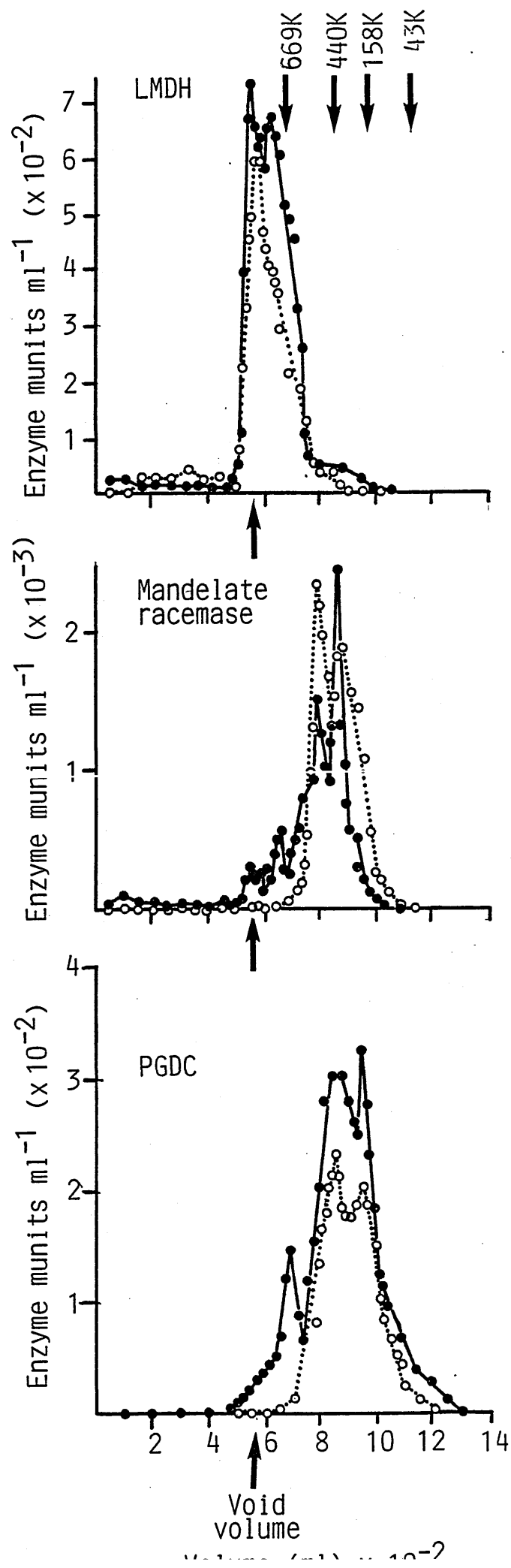
Halpin et al. (1981) also analysed sonic extracts from control and DMSI treated P.putida by gel filtration chromatography on Ultrogel AcA 22. Similar experiments were repeated (Figure 21) but Ultrogel AcA 34 was used because of its higher resolving power. Mandelate-racemase and PGDC elution profiles (Figure 21) from control extracts showed two peaks of activity, one corresponding to the reported M_r (280,000 and 90,000 respectively) and in the case of mandelate-racemase, a lower M_r peak (200,000) was observed whereas a higher M_r peak of PGDC activity (200,000) was observed. These multiple peaks of enzyme activity may be due to the buffer conditions used i.e. TNM buffer, pH8.0 which was used to allow comparison with the results reported by Halpin et al. (1981). The sharpness of the NAD-BDH peak (Figure 21) indicates that the column itself was satisfactory. All the enzymes assayed (except NAD-BDH) showed higher M_r species after DMSI treatment although only mandelate-racemase, PGDC and CO gave sharp peaks with high M_r values. With the exception of NAD-BDH, the enzymes eluted over a broad range of M_r values after DMSI treatment.

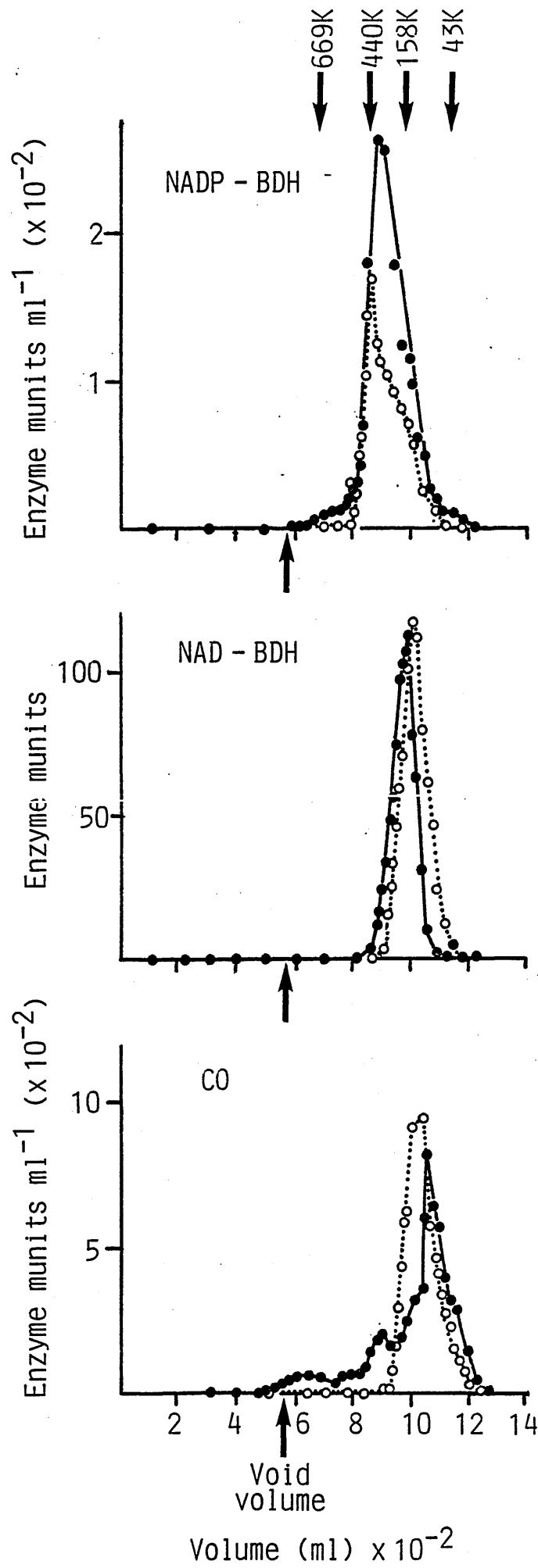
3.5.2 Crosslinking intact A.calcoaceticus

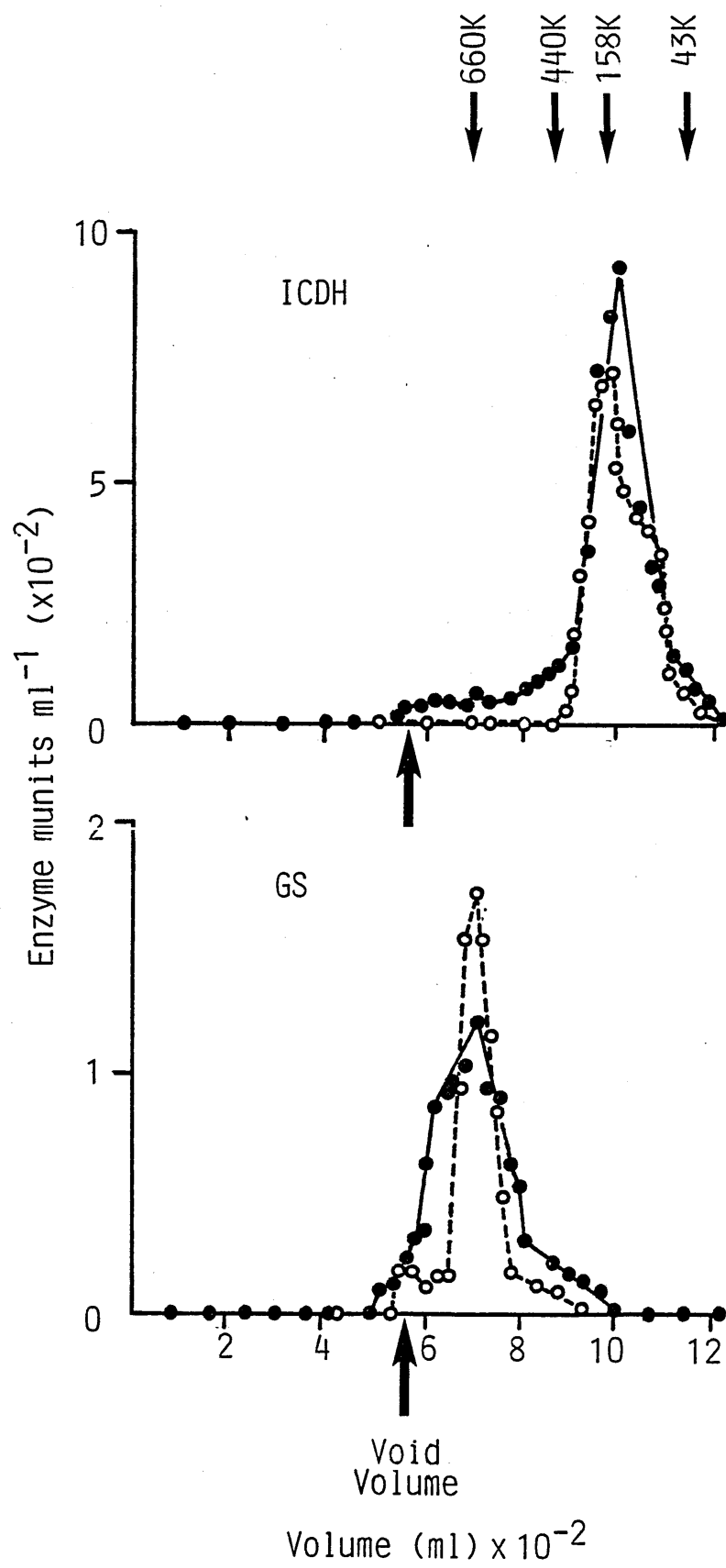
Although only non-specific crosslinking was observed in P.putida a series of experiments was carried out with A.calcoaceticus in the hope

Figure 21 Gel filtration chromatography of extracts from control and DMSI treated *P.putida*

Sonic extracts from control (o---o) and DMSI (●—●) treated bacteria (Methods 2.14.3) were applied to a 1.4l column (71cm long x 5.2cm diam.) of Ultrogel AcA 34 which had been equilibrated with TNM buffer, pH8.0. Enzyme activities were eluted by the same buffer at a flow rate of 60 ml h⁻¹. The column was calibrated with proteins of known M_r values and the elution position of four are indicated i.e. thyroglobin, $M_r = 669,000$; ferritin, $M_r = 440,000$; aldolase, $M_r = 158,000$ and ovalbumin, $M_r = 43,000$. The elution volume of Blue Dextran (approx. $M_r = 2,000,000$) was taken to indicate the void volume.







that specific crosslinking could be achieved.

A.calcoaceticus, mutant strain C1005, was treated with DMSI, disrupted by sonication and analysed by high speed centrifugation. The results obtained for A.calcoaceticus (Table 8) were similar to those for P.putida (Table 6) and LMDH (total specific activity) was inhibited by the DMSI treatment. There was an approx. 10 fold increase in the specific activity of PGDC recovered in the pellet and this corresponded to 1% of the total activity. However, as with P.putida the specific activities of the 'soluble' enzymes were also increased (Table 8) and the increase was observed only in exponentially growing bacteria.

Experiments which are summarised below were undertaken to try to achieve specific crosslinking (results not shown).

a) Different strains of A.calcoaceticus (strain 8250, mutant strains C1219 and C1123) gave similar results.

b) Different dimethylimidates (structures $R-(CH_2)_n-R$) i.e.

dimethylmalonimide (n=1); dimethylsuccinimide (n=2);

dimethylglutarimide (n=3); dimethyladipimide (n=4); DMPI (n=5)

and DMSI (n=6), all of which crosslink lysine residues, were studied.

In addition, the effect of PDM which crosslinks sulphhydryl groups was also investigated. No specific crosslinking was observed and in all cases LMDH (total specific activity) was inhibited. Slightly more of the 'soluble' enzymes (approx. 3% in the case of PGDC) were recovered in the pellet after crosslinking with DMPI than with any of the other crosslinkers.

c) Slightly more crosslinking (upto 6% PGDC associated with the pellet) was observed after bacteria had been treated with toluene in the presence of 8.5% (w/v) PEG (Methods 2.12). A 3-20 fold increase in the specific activities of the 'soluble' enzymes in the pellet was also observed in stationary phase bacteria after toluene/PEG treatment.

Table 8 Crosslinking *A.calcoaceticus* mutant strain C1005 intact bacteria with DMSI

Enzyme	Total specific activity (munits/mg protein)		Specific activity in pellet (munits/mg protein)		% recovered in pellet	
	Control	DMSI	Control	DMSI	Control	DMSI
LMDH	90	63	184	200	57	63
PGDC	166	166	0.7	8.0	0.11	1.0
BDH	86	79	ND	ND	ND	ND
CO	82	175	12	49	4.2	5.6
ICDH	157	166	6	27	3.5	3.2
GS	53	63	10	28	5.2	8.8
Protein	-	-	-	-	18	20

A.calcoaceticus mutant strain C1005, grown on complex media supplemented with D,L-mandelate, were harvested in late exponential phase and treated with DMSI in the presence of TNM buffer, pH8.0 (Methods 2.14.3). After disrupting the bacteria by sonication (Methods 2.9.1) the extracts were centrifuged at $105,000g_{av}$ (40K rpm, Ti50 rotor) for 60 min and the pellets were washed three times by resuspension/centrifugation. All the fractions were assayed for enzyme activity. ND = not detectable.

d) There was no evidence for specific crosslinking even in the presence of substrates and cofactors for the enzymes of the mandelate pathway. In these experiments bacteria were treated with toluene/PEG in an attempt to allow the substrates and cofactors to enter the bacteria.

3.5.3 Enzyme distribution in high speed centrifugation fractions after disruption of A.calcoaceticus by different methods

Since no specific crosslinking between the mandelate enzymes was observed it was necessary to investigate alternative methods of examining enzyme interactions. The effect of different methods of disruption on the enzyme distribution in high speed centrifugation fractions was studied.

a) Ultrasonic disruption

When bacteria were disrupted by sonication, 50-60% LMDH was recovered in the pellet fraction whereas 4% (or less) PGDC, BDH, CO and ICDH and approx. 30% GS was present in this fraction (Figure 22A).

b) Disruption by lysozyme treatment

When bacteria were disrupted by lysozyme treatment, 94% LMDH and 0-17% of the 'soluble' enzymes PGDC, BDH, CO and ICDH were recovered in the pellet fraction with 30% GS (Figure 22B).

c) Effect of number of times through the French pressure cell

Bacteria were disrupted by passing through the French pressure cell 1,2,3 or 4 times (Figure 23). When bacteria were passed through the French press once or twice, 97% LMDH, 40-55% PGDC, 3-15% BDH, CO and ICDH and 40% GS were recovered in the pellet fraction, whereas when bacteria were passed through the French press 3 or 4 times, 95% LMDH and approx. 20% PGDC still remained in the pellet with 3-8% BDH, CO and ICDH and 40% GS.

The presence of deoxyribonuclease or Mg^{++} did not alter these enzyme distributions. Approx. 30-40% GS sedimented irrespective of the method of disruption [this illustrated the behaviour of a high M_r

Figure 22 The effect of sonication and lysozyme treatment of
A.calcoaceticus on the enzymes recovered in the high
speed pellet

Bacteria (mutant strain C1005 grown on complex media supplemented with D,L-mandelate) were disrupted by sonication (Methods 2.9.1) or by lysozyme treatment (Methods 2.9.3.2) and the extracts (3ml) were centrifuged at $165,000g_{av}$ (50K rpm Ti50 rotor) for 150 min. The 'high speed pellet' so formed was washed twice by resuspending in 0.05M potassium phosphate buffer, pH7.5 to the original volume (3ml) and centrifuging. The results are expressed in terms of percentage of total enzyme activity recovered in the washed high speed pellet after sonication (A) and after lysozyme treatment (B). ND = not detectable.

enzyme activities in both samples were similar.

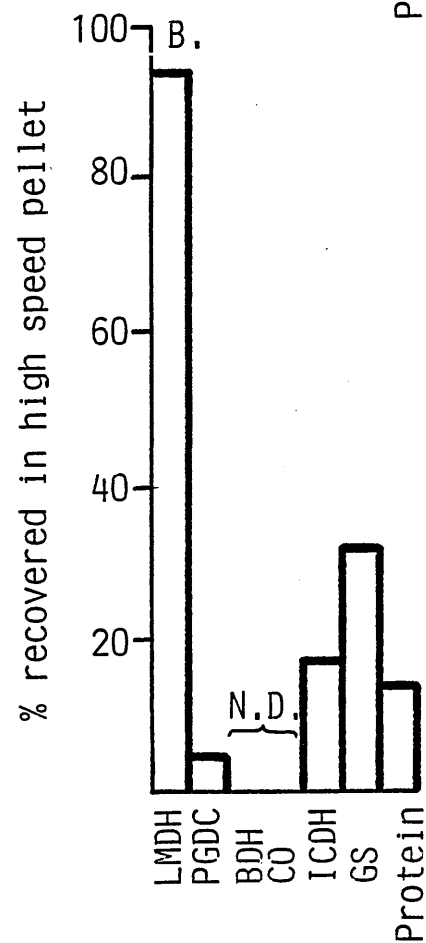
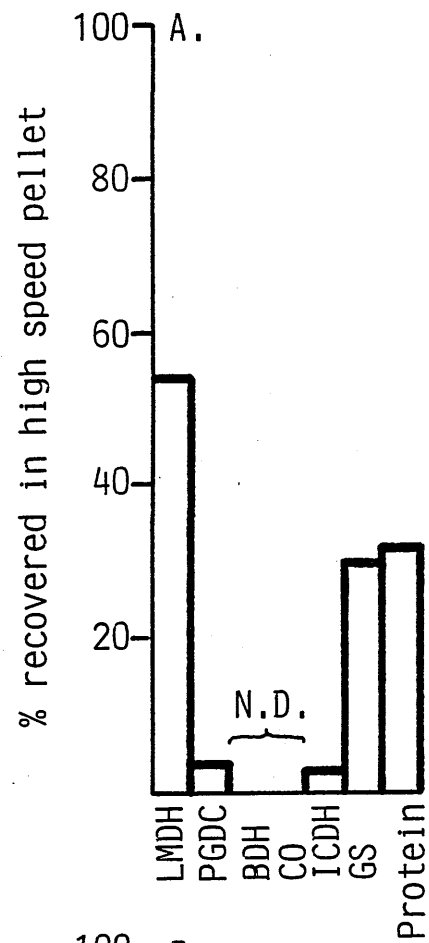
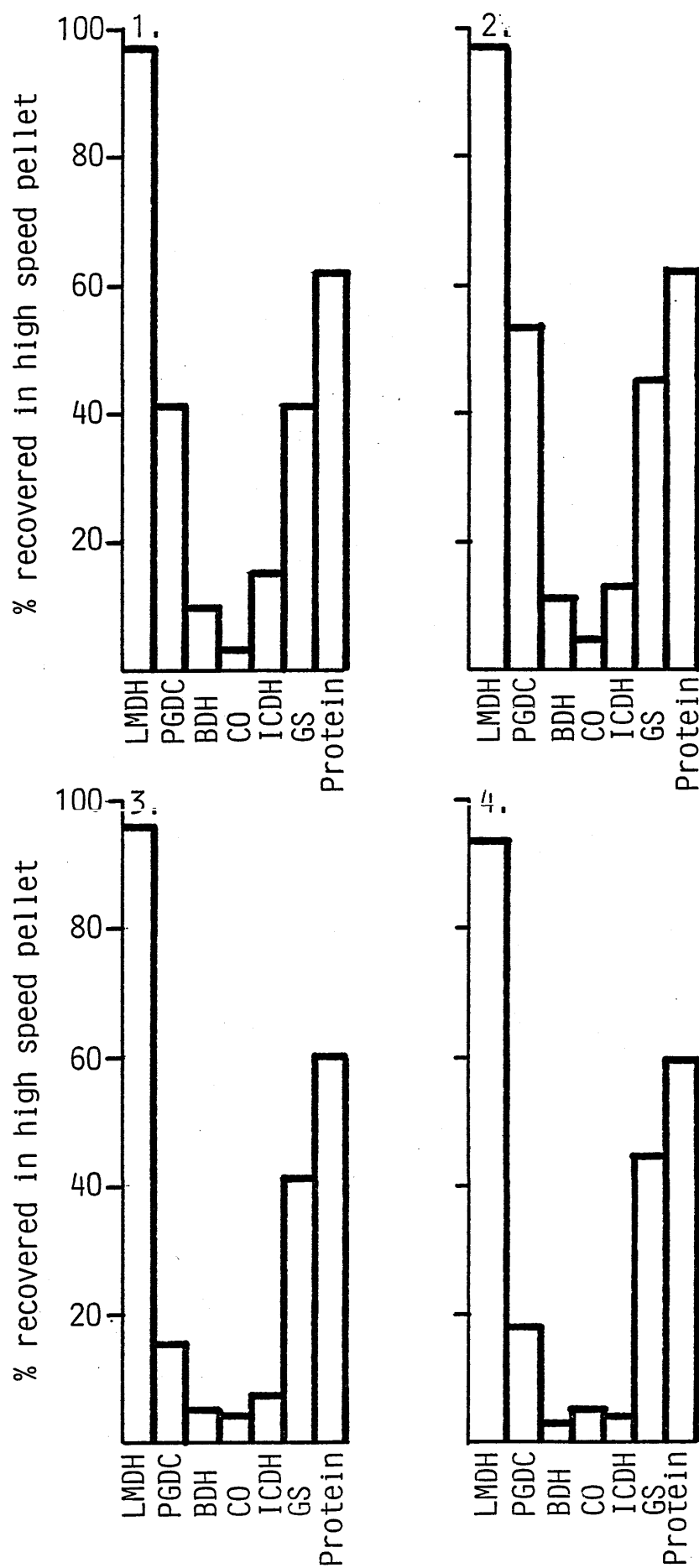


Figure 23 The effect of disrupting bacteria in the French pressure cell
on the enzymes recovered in the high speed pellet

Bacteria (mutant strain C1005 grown on complex media supplemented with D,L-mandelate) were disrupted by passing through the French pressure cell 1, 2, 3 or 4 times (Methods 2.9.2) and the extracts (3ml), were centrifuged at $165,000g_{av}$ (50K rpm, Ti50 rotor) for 150 min. The high speed pellets were washed twice by resuspending in 0.05M potassium phosphate buffer, pH7.5 to 3ml and centrifuging. The results are expressed in terms of percentage of total enzyme activity recovered in the washed high speed pellet.

The total enzyme activities in all samples were similar.



protein (600,000) under the conditions of centrifugation used].

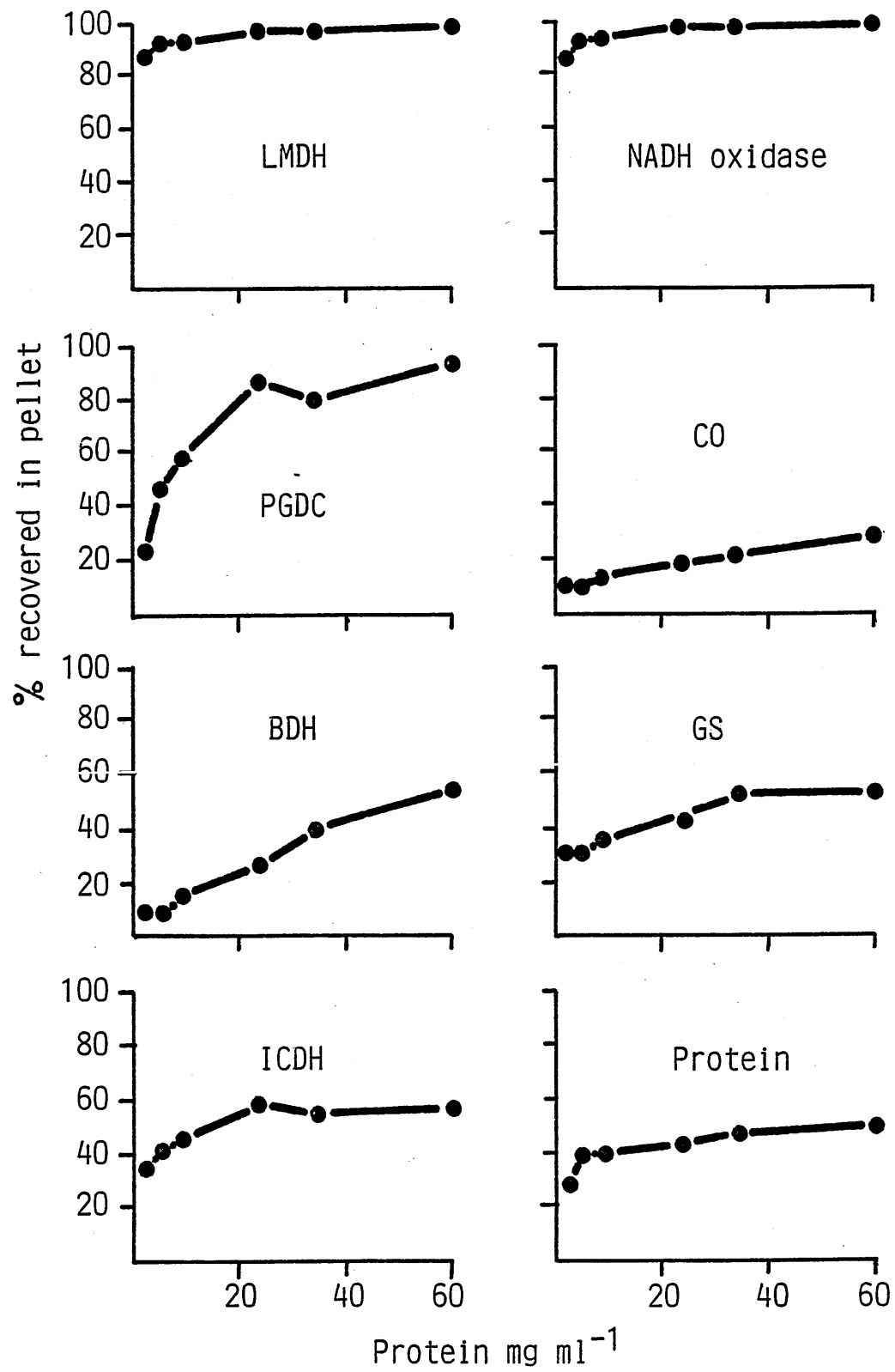
3.5.4 Effect of protein concentration on enzyme sedimentation

Since the protein concentration may be important for enzyme interactions, an experiment was undertaken to investigate this variable. In the previous experiment (Figure 23), the high speed pellets were washed by resuspending to the original volume (i.e. 3ml). As 50-60% of the total protein was recovered in the pellet fraction, this procedure involved dilution and so in the following study the pellets were washed by resuspending to half the original volume (i.e. 1.5ml) in an effort to maintain the high protein concentration.

The protein concentration of the French press homogenate had little effect on the amount of LMDH and NADH oxidase recovered in the pellet (Figure 24); approx. 98% of these two enzymes sedimented at the highest protein concentration (60mg ml^{-1}) and 86% at the lowest protein concentration (3mg ml^{-1}). However, the protein concentration had a remarkable effect on the amount of PGDC recovered in the pellet; at a protein concentration of 60mg/ml^{-1} , 92% PGDC was recovered in the pellet fraction, whereas at a protein concentration of 3mg ml^{-1} only 22% PGDC was recovered in this fraction. The distribution of the other enzymes also varied with the protein concentration of the French press homogenate (from highest to lowest protein concentration), BDH, 56 to 9%; ICDH, 58 to 34%; CO, 30 to 9%; GS, 54 to 32% and protein, 50 to 30%. These results may illustrate a specific interaction of PGDC with the membrane and therefore possibly with LMDH at high protein concentrations. If the amount of ICDH (58%) recovered in the pellet fraction is taken as a measure of non-specific binding, then possibly, 34% of the total PGDC interacts specifically (at a protein concentration of 60mg ml^{-1}) with the membrane fraction. Bacteria resuspended to 1g ml^{-1} were difficult to handle and so in future experiments bacteria

Figure 24 The effect of the protein concentration of the French press homogenate on the enzymes recovered in the high speed pellet

Bacteria (mutant strain C1005 grown on complex media supplemented with D,L-mandelate) were washed once in 0.05M potassium phosphate buffer, pH7.5 and resuspended to a concentration of (g wet wt ml⁻¹) 1, 0.5, 0.25, 0.125, 0.0625 and 0.03 in the same buffer. The bacteria were disrupted by two passages through the French pressure cell (Methods 2.9.2) and the protein concentration of the resulting French press homogenates was determined. The homogenates (3ml) were centrifuged at 165,000g_{av} (50K, Ti50 rotor) for 150 min and the high speed pellets were washed twice by resuspending to 1.5ml and centrifuging. The percentage of the total enzyme activity recovered in the high speed pellet is plotted against the protein concentration of the homogenate.



were resuspended to 0.5gml^{-1} which resulted in French press homogenates with a protein concentration of $30\text{-}40\text{mg ml}^{-1}$.

3.5.5 The effect of disrupting bacteria in different buffers

The buffer system used in the previous French press experiments (Results 3.5.3 & 3.5.4) was 0.05M potassium phosphate buffer, pH7.5. Some loss of PGDC activity was observed when assays were carried out on successive days and so it was necessary to investigate different buffer systems with a view to stabilising PGDC activity. The buffer systems tested are shown in Table 9, and the effect of addition of 100 μM TPP, 100 μM DTT, 2mM MgSO_4 and 1mM EDTA was studied. These reagents were chosen since it had been necessary to include them in all buffers used during PGDC purification (Barrowman & Fewson, 1985).

LMDH activity was not much affected by the presence of TPP, DTT, MgSO_4 and EDTA but the specific activity of LMDH was always lower at pH6.0. The highest LMDH specific activity was obtained with 0.02M potassium phosphate buffer, pH7.5. The highest total PGDC specific activity was obtained with either 0.05M potassium phosphate buffer, pH6.0 or 0.1M potassium phosphate buffer, pH7.5 both in the presence of TPP, DTT, MgSO_4 and EDTA. In all cases the presence of TPP, DTT, MgSO_4 and EDTA resulted in higher total specific activities of PGDC. The total specific activity of ICDH was variable and showed no particular pattern.

In all cases (except 0.02M potassium phosphate buffer, pH7.5) the presence of TPP, DTT, MgSO_4 and EDTA resulted in a higher PGDC specific activity in the pellet with 0.1M potassium phosphate buffer, pH7.5 containing TPP, DTT, MgSO_4 and EDTA giving the highest PGDC specific activity in the pellet.

A very high percentage (80%) of protein was recovered in the pellets at 0.02M potassium phosphate buffer, pH6.0 which would account for the low specific activities of PGDC and ICDH in the pellets in the presence

Table 9 The effect of disrupting bacteria in different buffers

Bacteria (mutant strain C1005 grown on complex media supplemented with D,L-mandelate) were harvested and washed once in one of the buffers listed, resuspended to 0.5g wet wt ml⁻¹ in the appropriate buffer and disrupted by passing through the French press twice (Methods 2.9.2). The homogenates (3ml) were centrifuged at 165,000g_{av} (50K rpm, Ti50 rotor) for 150 min and the pellets were washed twice with the appropriate buffer by resuspending to 1.5ml and centrifuging. Each fraction was assayed for enzyme activity.

Potassium phosphate concentration	Buffer pH	100μM TPP, 100μM DTT 2mM MgSP ₄ , 1mM EDTA	Total specific activity (munits/mg protein)			Specific activity in pellet (munits/mg protein)			% recovered in pellet			
			LMDH	PGDC	ICDH	LMDH	PGDC	ICDH	LMDH	PGDC	ICDH	Protein
0.05M	7.5	-	210	680	438	516	1104	500	97	64	45	39
0.05M	7.5	+	237	828	578	563	1584	582	98	79	42	41
0.05M	6.0	-	201	802	670	360	1028	492	95	68	39	53
0.05M	6.0	+	197	1183	604	344	1695	412	97	79	38	55
0.1M	7.5	-	280	810	742	552	798	399	93	47	25	47
0.1M	7.5	+	253	1062	747	522	1765	588	98	79	38	48
0.1M	6.0	-	121	270	332	495	882	552	99	82	40	44
0.1M	6.0	+	193	907	859	353	1384	592	98	80	37	45
0.02M	7.5	-	283	968	674	496	1350	442	99	78	37	56
0.02M	7.5	+	302	901	876	579	1365	636	98	77	37	57
0.02M	6.0	-	172	286	450	213	305	288	99	86	56	80
0.02M	6.0	+	202	621	483	279	756	310	99	87	46	72

of this buffer (Table 9). This is probably due to a high degree of non specific binding at this concentration of phosphate and pH. Except for this buffer, the percentage of protein recovered in the pellet was more or less constant at 40-50%.

The percentage of LMDH recovered in the pellet was unaffected by the buffer conditions whereas the percentage of PGDC recovered in the pellet varied from 47% (0.1M potassium phosphate buffer, pH7.5) to 87% (0.02M potassium phosphate buffer, pH6.0 containing TPP, DTT, MgSO_4 and EDTA). The percentage of ICDH recovered in the pellet varied from 25-56%. In order to maximise both LMDH and PGDC specific activities and the percentage of PGDC in the pellet, 0.1M potassium phosphate buffer, pH7.5 containing 100 μM TPP, 100 μM DTT, 2mM MgSO_4 and 1mM EDTA was the buffer chosen to use in future experiments.

3.5.6 The effect on enzymes recovered in the pellet of different washing conditions

High speed pellets were washed with various solutions described in Table 10. It was hoped that a washing condition would be found which would preferentially wash ICDH from the pellets while leaving PGDC bound to the membranes.

In all cases 70-73% of the total PGDC activity was recovered in the pellet before washing and the total specific activities of the three enzymes remained constant irrespective of the washing condition. MgCl_2 and EDTA had no effect on the percentage of PGDC and ICDH recovered in the pellets whereas after washing with NaCl, KCl or buffer at pH6.0 (Table 10), a further 10-18% of the total PGDC was washed from the membrane (compared with the control) leaving approx. 60% of the total PGDC still associated with the membrane fraction. EGTA treatment washed approx. 30% more PGDC from the pellet. Molar potassium phosphate at pH6.0, 7.5 and 8.5 washed a further 30% ICDH from the high speed pellet leaving 12-19% ICDH still associated with this fraction. Molar phosphate also washed more PGDC from the membrane leaving only 18-35% PGDC associated

Table 10 The effect of washing the high speed pellet with
different conditions

	% of total recovered in pellet			
	LMDH	PGDC	ICDH	Protein
Buffer A	97	73	38	40
Buffer A + 0.2M NaCl	97	57	34	40
Buffer A + 0.5M KCl	97	63	37	38
Buffer A + 0.2M MgCl ₂	97	72	32	39
Buffer A + 0.02M EDTA	96	75	39	40
Buffer A + 0.02M EGTA	96	44	35	36
Buffer A but with 1M potassium phosphate, pH6.0	92	35	18	36
Buffer A but with 1M potassium phosphate	87	28	19	29
Buffer A but with 1M potassium phosphate, pH8.5	81	18	12	24
Buffer A but at pH6.0	94	55	35	43
Buffer A but at pH8.5	96	68	46	45

Bacteria (mutant strain C1005 grown on complex media supplemented with D,L-mandelate) were harvested and washed once with Buffer A (i.e. 0.1M potassium phosphate buffer, pH7.5 containing 100 μ M TPP, 100 μ M DTT, 2mM MgSO₄ and 1mM EDTA) and resuspended to 0.5g wet wt ml⁻¹ in the same buffer. The bacteria were disrupted by two passages through the French pressure cell (Methods 2.9.2) and the resulting homogenates (11 x 3ml) were centrifuged at 165,000g_{av} (50K rpm, Ti50 rotor) for 150 min. The pellets were resuspended to 1.5ml in one of the listed buffers (all of which contained 100 μ M TPP, 100 μ M DTT, 2mM MgSO₄ and 1mM EDTA) and incubated for 30 min at 4°C after which the high speed centrifugation was repeated. Results are expressed in terms of percentage of total recovered in the pellet after washing.

with this fraction. The amount of ICDH recovered in the pellet was unaffected by the other washing conditions (Table 10).

These results were rather surprising since the conditions used are methods well established for washing extrinsic proteins from membranes and so it was expected that more ICDH would have been washed from the pellets under these conditions. It may be that more exhaustive washing conditions were required.

In an attempt to wash the high speed pellets more thoroughly, they were washed with increasing concentrations of NaCl by resuspending in the appropriate buffer to half the starting volume (1.5ml) or to 2.5 times the starting volume (7.5ml) (Table 11). By increasing the NaCl concentration in the wash to 1.0M approx. 20% more ICDH and PGDC were washed from the pellet leaving 19% and 52% respectively still associated with the membrane fraction (Table 11A). When the pellet was washed with 2.5 times the starting volume of the control buffer (Table 11B), a further 50% of PGDC and 40% ICDH was washed from the pellet, leaving 23% and 7% respectively still associated with the membrane fraction. Washing with 2.5 times the starting volume of 0.2M NaCl and 0.5M NaCl did not release more PGDC compared with the control but 1.0M NaCl resulted in a further 10% PGDC being washed from the high speed pellet (Table 11B).

That more PGDC can be washed from the membrane by diluting the pellet (by washing in 2.5 times the starting volume) is consistent with the observation that the association of PGDC with the membrane is dependent on a high protein concentration (Figure 24). In all these experiments (Results 3.5.3-3.5.6) with French press homogenates, there was always more PGDC (6-40%) than ICDH associated with the membranes and so experiments were carried out to investigate the nature of the association of PGDC with the membrane fraction.

3.5.7 Analysis of French press homogenates by Percoll gradient centrifugation

A Percoll gradient was chosen such that the membranes would sediment

Table 11 The effect of washing high speed pellets with different buffer volumes and increasing NaCl concentrations

1. Wash	% of total recovered in pellet			
	LMDH	PGDC	ICDH	Protein
Buffer A	93	76	47	43
Buffer A + 0.2M NaCl	92	70	38	42
Buffer A + 0.5M NaCl	92	63	26	36
Buffer A + 1.0M NaCl	92	52	19	29

2. Wash	% of total recovered in pellet			
	LMDH	PGDC	ICDH	Protein
Buffer A	94	23	7.5	25
Buffer A + 0.2M NaCl	95	23	7.2	23
Buffer A + 0.5M NaCl	96	22	4.6	20
Buffer A + 1.0M NaCl	94	12	3.2	20

Bacteria (mutant strain C1005 grown on complex media supplemented with D,L-mandelate) were washed and resuspended to $0.5\text{g wet wt ml}^{-1}$ in Buffer A (i.e. 0.1M potassium phosphate buffer, pH7.5 containing 100 μM TPP, 100 μM DTT, 2mM MgSO_4 and 1mM EDTA) and disrupted by two passages through the French pressure cell (Method 2.9.2). The resulting homogenates (8 x 3ml) were centrifuged at $165,000g_{av}$ (50K rpm, Ti50 rotor) for 150 min. The pellets were washed twice by resuspending to either 1.5ml (1) or 7.5ml (2) in Buffer A or Buffer A containing 0.2, 0.5 or 1.0M NaCl.

in the Percoll gradient in situ leaving the soluble protein in the buffer layer above the gradient. The enzyme profiles obtained when a French press homogenate was subjected to density gradient centrifugation are illustrated in Figure 25.

LMDH and NADH oxidase activities peaked at a density of approx. 1.05 whereas PGDC, ICDH and CO remained in the buffer layer with only some activity sedimenting to positions of higher density. There was no evidence of co-migration of LMDH and PGDC. Essentially similar profiles were obtained when high speed pellets and supernatants were treated in the same way (results not shown). That no co-migration of LMDH and PGDC was observed may have been due to dilution of the sample in the Percoll solution and so a similar experiment was carried out where the Percoll gradient was run in the presence of a high speed supernatant ($30\text{mg protein ml}^{-1}$) from a non-induced strain (8250) which had been grown on complex media. This was to try to mimic the conditions in the bacteria as far as possible with a view to protein concentration, pH and ionic strength. However, a similar enzyme profile was observed (results not shown).

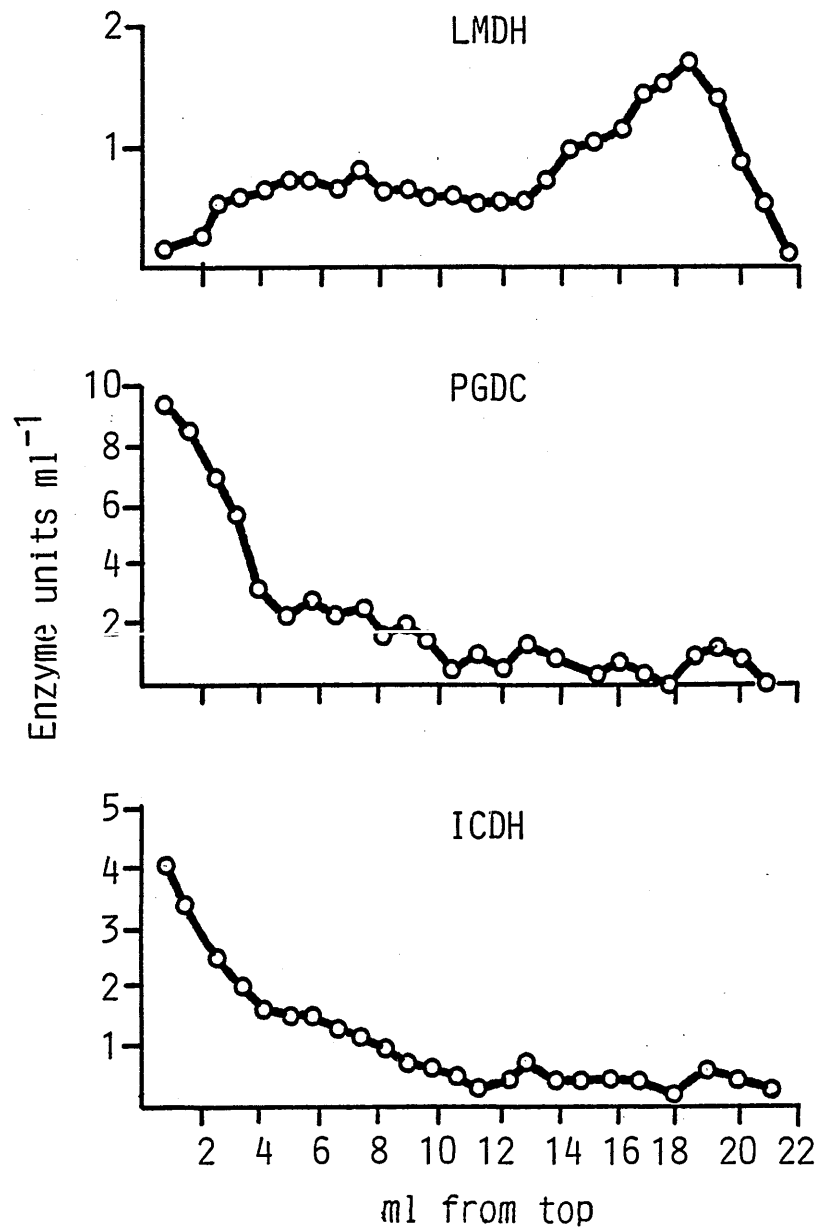
3.5.8 Gel filtration chromatography of a French press homogenate

A French press homogenate (Figure 26) and high speed pellets (results not shown) were analysed by gel filtration chromatography. Essentially the same elution profiles were obtained with these two samples. LMDH eluted close to the void volume whereas PGDC and ICDH eluted at a position corresponding to a lower apparent M_r (although these two enzymes were not separated on this column). No co-elution of PGDC with the LMDH activity was observed.

There was the possibility that LMDH and PGDC associate in some way but may have dissociated due to dilution during the gel filtration chromatography. With this in mind, experiments were carried out whereby the column was equilibrated and run in the presence of BSA (30mg ml^{-1}) or in the presence of 20% (v/v) glycerol. However, no

Figure 25 Percoll density gradient centrifugation of a French press
homogenate

After bacteria (mutant strain C1005 grown on complex media supplemented with D,L-mandelate) had been disrupted by two passages through the French pressure cell (Methods 2.9.2), the resulting homogenate was layered on a solution of 20% Percoll and density gradient centrifugation was carried out (Method 2.16). The gradient was calibrated by density gradient marker beads and 1ml fractions were collected from the top of the tube and assayed for enzyme activity and protein.



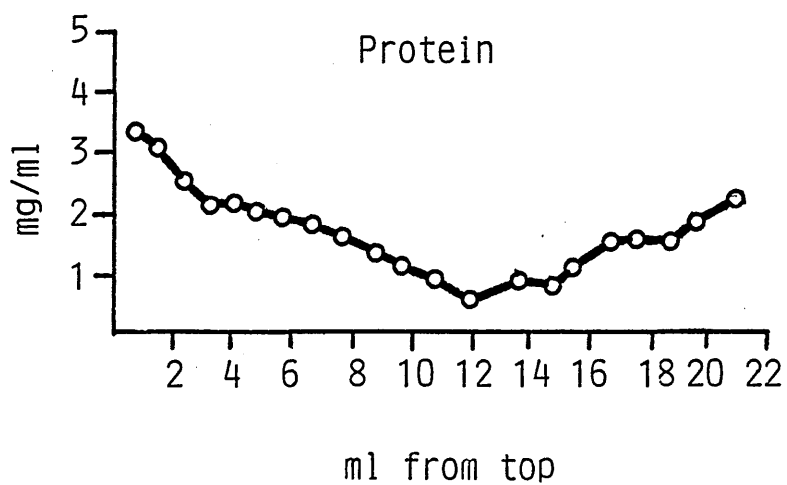
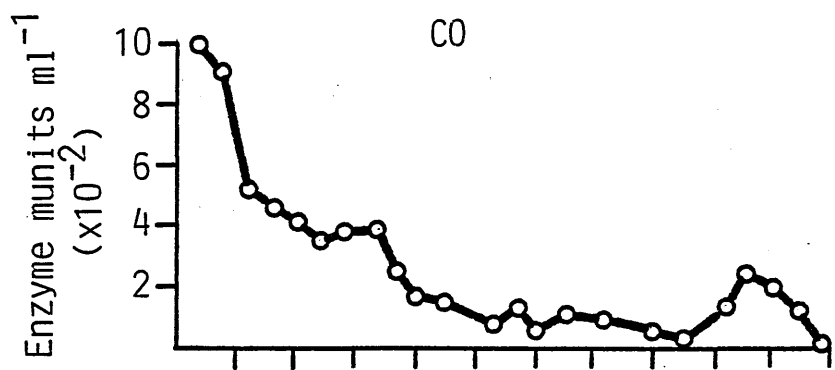
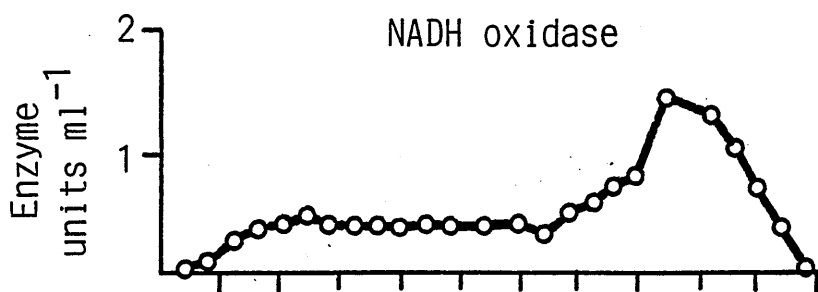
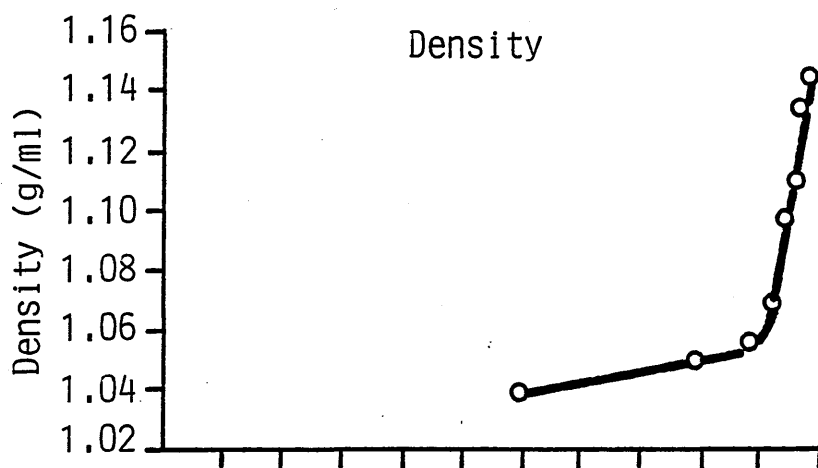
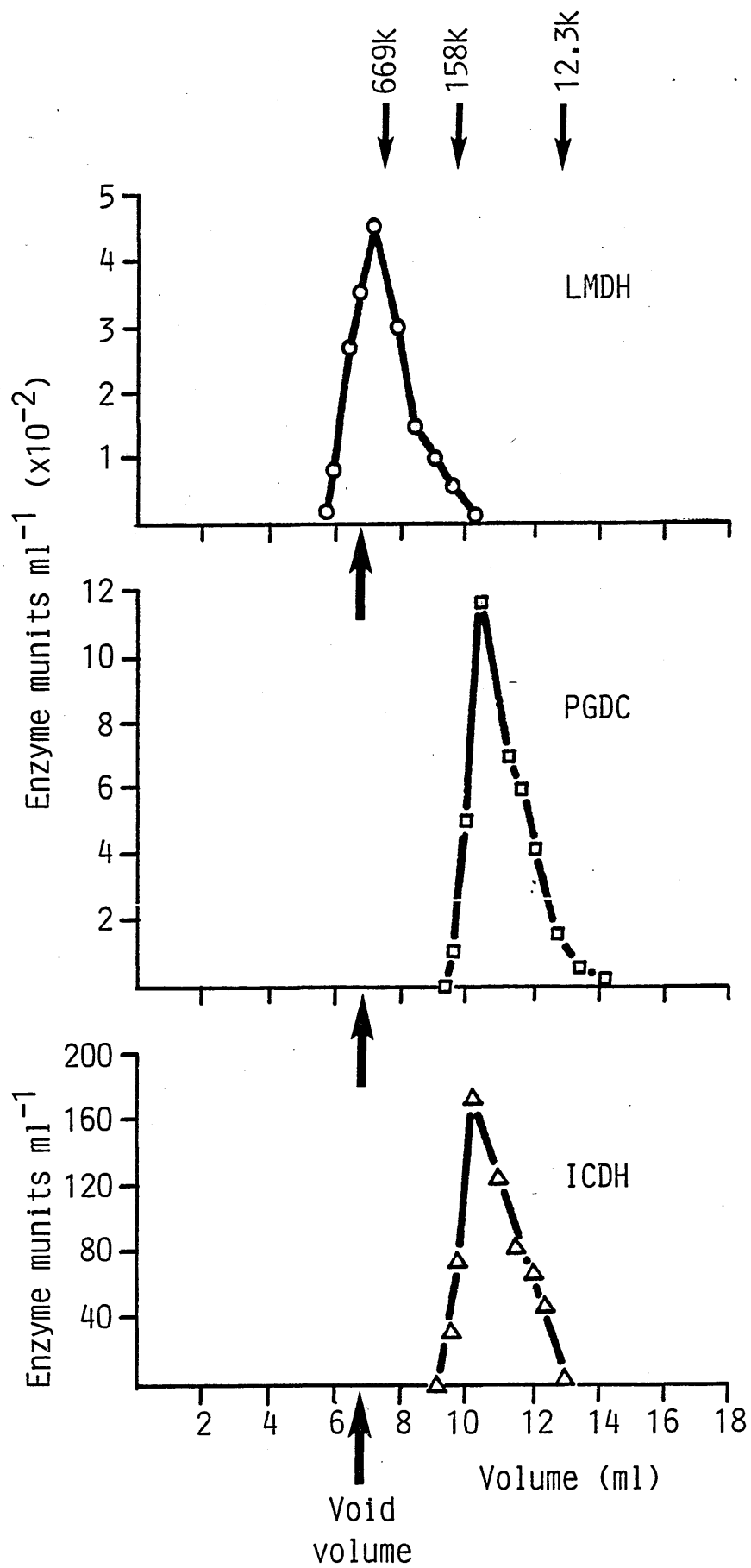


Figure 26 Gel filtration chromatography of a French press homogenate

Bacteria (mutant strain C1005 grown on complex media supplemented with D,L-mandelate) were disrupted by passing through the French pressure cell twice (Methods 2.9.2) and the resulting homogenate was applied to a column (30cm long x 0.7cm diam.) of Ultrogel AcA 34 equilibrated with 0.1M potassium phosphate buffer, pH7.5 containing 100 μ M TPP, 100 μ M DTT, 2mM MgSO₄ and 1mM EDTA. Enzyme activity was eluted with the same buffer at a flow rate of 4ml h⁻¹ and fractions were collected every 7.5 min. The column was calibrated with proteins of known M_r values and the elution positions are indicated i.e. thyroglobulin, M_r = 669,000; aldolase, M_r = 158,000 and cytochrome c, M_r = 12,300. The elution volume of Blue Dextran (approx. M_r = 2,000,000) was taken to be the void volume.



evidence for the co-elution of LMDH and PGDC was observed (results not shown).

3.5.9 The effect of PEG on the association of PGDC with the membrane fraction

PEG is thought to promote specific enzyme interactions by mimicking the high protein concentration in the cell (e.g. Halper & Srere, 1977) and so the effect of PEG on the association of PGDC with the membrane fraction was studied. French press homogenates were prepared from bacteria which had been resuspended in the presence or absence of 14% (w/v) PEG (M_r 6,000) and the homogenates were centrifuged at $165,000g_{av}$ (50K rpm, Ti50 rotor) for 150 min. In the control samples (no PEG present) approx. 70% PGDC was recovered in the high speed pellet; by contrast only 35% PGDC was recovered in the pellet fraction when PEG was present and so it can be concluded that PEG interferes with, rather than promotes the association of PGDC with the membrane.

3.5.10 Development of methods for crosslinking French press homogenates with dimethylimidates

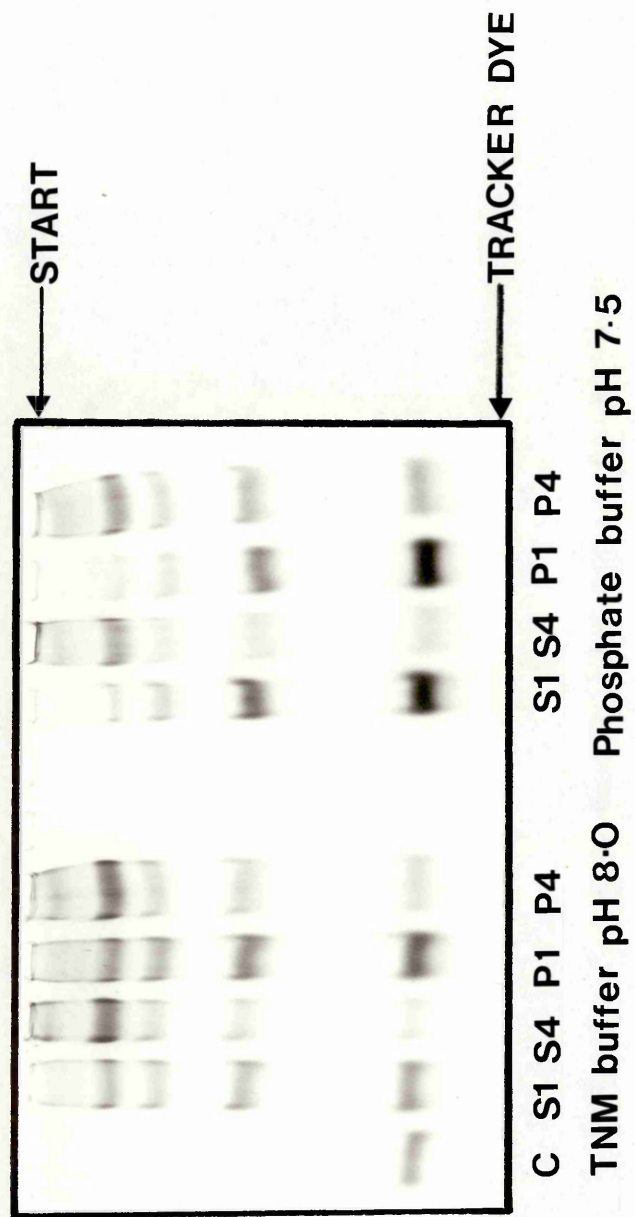
Since such a high percentage (approx. 70%) of PGDC can be associated with the membrane fraction after disruption of the bacteria in the French pressure cell, it was thought that by crosslinking the French press homogenate, any enzyme interaction might be trapped and so facilitate the study of such interactions. The earlier crosslinking experiments in vivo (Results 3.5.2) were carried out in the presence of TNM buffer, pH8.0; however the PGDC tetramer is known to dissociate at this high pH (Barrowman, 1981) and so a method for crosslinking with the dimethylimidates at a lower pH was developed.

a) Crosslinking aldolase

The dimethylimide crosslinkers (DMSI and DMPI) require a high pH (\geq pH8.0) for maximal activity. An experiment was carried out comparing the crosslinking of aldolase by DMSI and DMPI in the presence of TNM buffer, pH8.0 and in the presence of phosphate buffer, pH7.5 (Figure 27).

Figure 27 Crosslinking of aldolase

Aldolase was treated with the crosslinking reagents DMSI (S) and DMPI (P) in the presence of TNM buffer, pH8.0 or phosphate buffer, pH7.5 (Methods 2.14.2) with either 1 (S1,P1) or 4 (S4,P4) additions of the crosslinker over the 60 min incubation period. The samples were run on 5% phosphate SDS polyacrylamide gels (Methods 2.21.1) which were stained with Coomassie Blue. Approx. 5 μ g of protein was applied to each track. C = control.



Crosslinking of aldolase took place after one addition of the reagents (DMSI and DMPI) in the presence of phosphate buffer, pH7.5 but less trimer and tetramer were formed than when the reaction was carried out at pH8.0 (Figure 27). However, when DMSI and DMPI were added 4 times over the 60 min incubation period (to compensate for the short half life of the reagents), an equivalent amount of crosslinking was observed at pH7.5 as that obtained with one addition of the reagents at pH8.0. This showed that crosslinking with the dimethylimidates was possible at pH7.5.

b) Crosslinking the French press homogenate

Freshly harvested bacteria (mutant strain Cl005 grown on complex media supplemented with D,L-mandelate) were treated with DMSI and DMPI during the preparation of French press homogenates (Methods 2.14.4). After incubating with the crosslinker (which was added every 15 min for 1h) the French press homogenates were subjected to high speed centrifugation (Methods 2.11.2).

There was no increase in the amount of PGDC recovered in the pellet fraction after treating with DMSI or DMPI. When the pellets were washed by resuspending to half the original volume of the homogenate, approx. 70% of the total PGDC and 40% ICDH was recovered in the high speed pellets of control and treated samples. When the pellets were washed by diluting to 2.5 times the original volume, 36% PGDC and 6% ICDH remained associated with the membrane fractions of control samples whereas 44% PGDC and 6% ICDH was recovered in the pellet fraction of the crosslinked homogenate.

c) Analysis of crosslinked French press homogenates by immunoblotting

Control and crosslinked French press homogenates were analysed by immunoblotting with anti-LMDH, anti-DMDH and anti-PGDC as described in Figure 28.

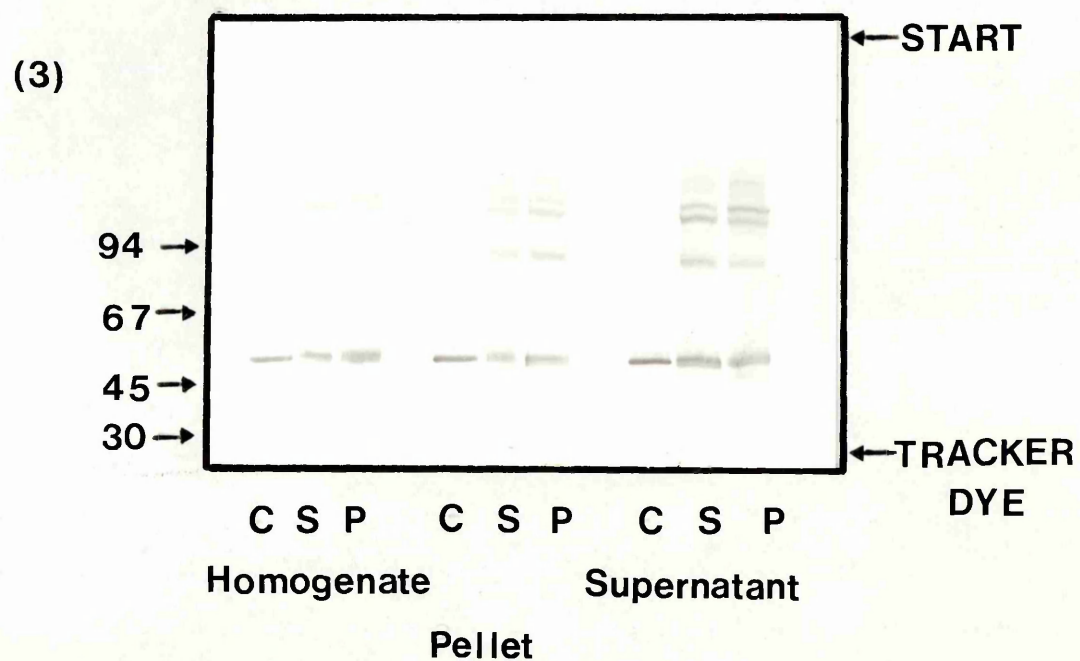
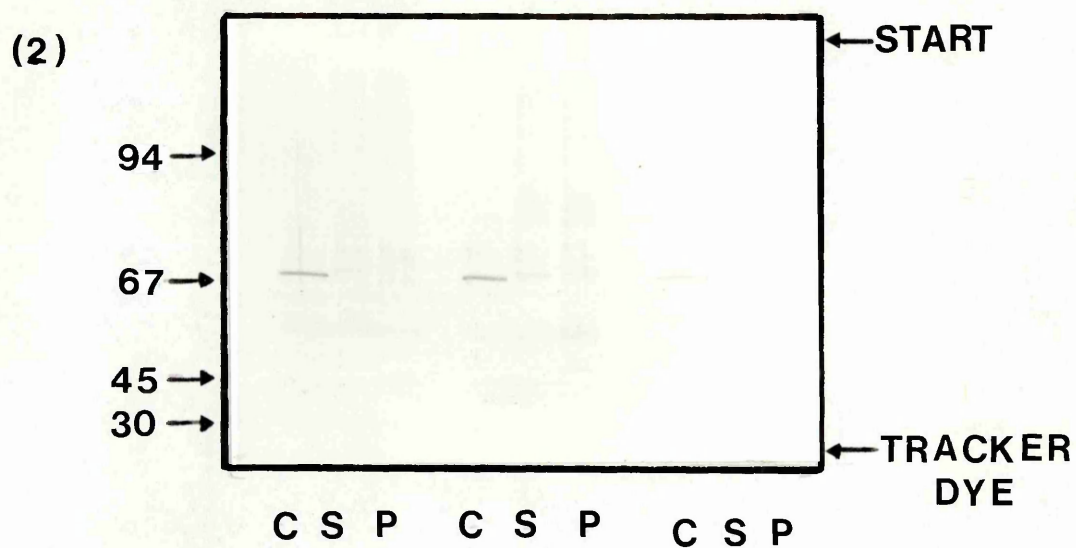
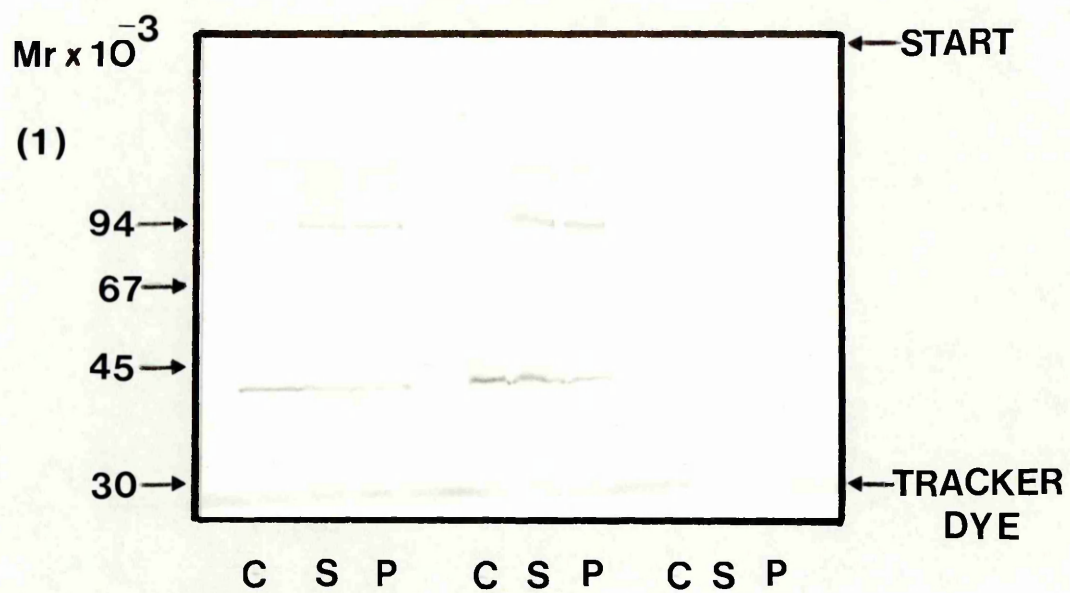
Figure 28 Immunoblots of control and crosslinked extracts

French press homogenates (prepared from mutant strains C1005 and C1123 grown on complex media supplemented with D,L-mandelate) were treated with DMSI and DMPI (Methods 2.14.4) in the presence of 0.05M potassium phosphate buffer, pH7.5. The homogenates were centrifuged at $165,000g_{av}$ (50K rpm, Ti50 rotor) for 150 min and the pellets, supernatants and French press homogenates were run on 7.5% SDS polyacrylamide gels and subsequently immunoblotted (Methods 2.22) with anti-LMDH, anti-DMDH and anti-PGDC.

- (1) fractions from strain C1005 blotted with anti-LMDH
- (2) fractions from strain C1123 blotted with anti-DMDH
- (3) fractions from strain C1005 blotted with anti-PGDC

C = control; S = DMSI; P = DMPI

The positions of Pharmacia molecular mass standards which had been stained for protein (not shown) are indicated.



In the control (uncrosslinked) samples there were bands corresponding to LMDH, DMDH or PGDC whereas in the cross-linked samples there were bands with high apparent M_r values. In all cases DMSI and DMPI gave similar results. In the anti-LMDH blot (Figure 28.1) there were bands with apparent M_r values of 140,000 (this band was not always detected and sometimes appeared in the control sample), 100,000 and 40,000 (equivalent to LMDH). In the anti-DMDH blot (Figure 28.2), there was a band with an apparent M_r value of 66,000 (equivalent to DMDH) in the control sample; however there was no band with this M_r value in the crosslinked sample but there was one with a slightly higher M_r value (68,000). This may be due to intra-molecular crosslinking of the DMDH. In addition there was a smear in the crosslinked samples which may indicate that DMDH was crosslinked to species of various M_r values. In the anti-PGDC blot (Figure 28.3) bands with apparent M_r values of 135,000, 120,000, 98,000 and 56,000 (equivalent to PGDC) were detected. A similar blotting pattern with anti-PGDC was obtained with extracts from mutant strain Cl005 (LMDH and PGDC activities present) and mutant strain Cl123 (DMDH and PGDC activities present). PGDC is reported to be a tetramer (Barrowman & Fewson, 1985), however there was no evidence for trimers or tetramers of PGDC being formed on crosslinking. The band with an apparent M_r of 120,000 may be a dimer of PGDC.

3.5.11 Crosslinking a French press homogenate from mutant strain Cl408 and a washed 'wall + membrane' fraction from mutant strain Cl005

There was a band with an apparent M_r of 100,000 on the anti-LMDH blot of the crosslinked sample and a band with an apparent M_r of 98,000 on the anti-PGDC blot of the same sample. If there was an LMDH-PGDC crosslinked species this would have an expected M_r of 102,000 (by addition of the reported M_r values of LMDH and PGDC i.e. 44,000 and 58,000 respectively). The following experiment was carried out to investigate the possibility that the band with an approx. M_r value of 100,000 was a crosslinked hybrid

of LMDH and PGDC. French press homogenates from mutant strain C1005 (LMDH and PGDC present) and from mutant strain C1408 (no LMDH activity; PGDC activity present) and a 'wall + membrane' fraction from mutant strain C1005 (LMDH activity, no PGDC activity present) were treated with DMSI and immunoblotted with anti-LMDH and anti-PGDC (Figure 29).

The crosslinked French press homogenate from mutant strain C1408 showed a similar pattern to that from mutant strain C1005 in the anti-PGDC blot (Figure 29B) even although there was no positive band in the anti-LMDH blot of the C1408 sample (Figure 29A). The crosslinked washed 'wall + membrane' fraction showed a similar pattern to the French press homogenate of mutant strain C1005 in the anti-LMDH blot even although there was no positive band in the anti-PGDC blot of the washed 'wall + membrane' sample from C1005. It can therefore be concluded that LMDH and PGDC were crosslinked to other protein species but not to each other.

3.5.12 Gel filtration chromatography of control and DMSI treated extracts

Control and DMSI treated French press homogenates were analysed by gel filtration chromatography (Figure 30). In samples treated with DMSI, there were two peaks of 'soluble' enzyme activity, one corresponding to the control activity and a second species with a higher M_r which eluted close to the void volume. LMDH activity eluted in the void volume in both control and DMSI treated samples. These results confirm the non-specificity of the crosslinking reaction since high M_r species, co-eluting with LMDH activity, were obtained with the control enzymes (CO and ICDH) as well as the 'soluble' mandelate enzymes.

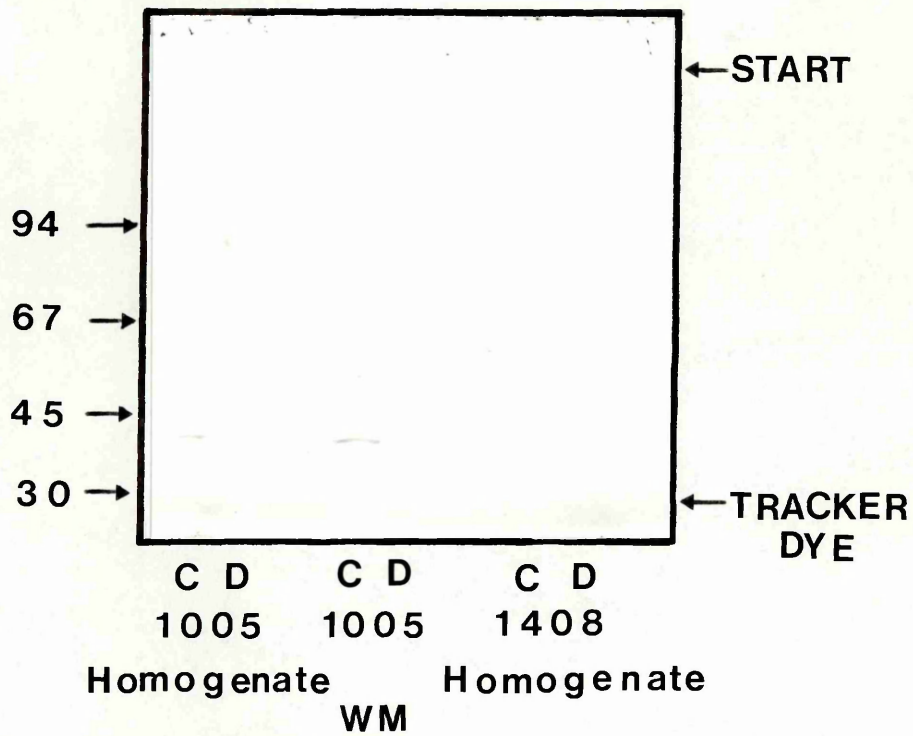
3.5.13 Confirmation of crosslinking in vivo

Confirmation that the crosslinking reagent DMSI can penetrate exponential phase bacteria (mutant strain C1005) was obtained by subjecting sonic extracts from control and DMSI treated bacteria (in the

Figure 29 Immunoblots of control and crosslinked homogenates from mutant strains C1005 and C1408 and of a crosslinked 'wall + membrane' fraction from mutant strain C1005

Bacteria (mutant strain C1005 grown on complex media supplemented with D,L-mandelate) were disrupted by sonication (Methods 2.9.1) and a 'wall + membrane' fraction was prepared (Methods 2.11.2) and washed thoroughly until no PGDC activity was detected. This 'wall + membrane' fraction and French press homogenates from strains C1005 (grown on complex media supplemented with D,L-mandelate) and C1408 (grown on complex media supplemented with phenylglyoxylate) were treated with DMSI (Methods 2.14.4) and immunoblotted (Methods 2.22) with anti-LMDH (A) or anti-DMDH (B). C = control; D = DMSI; WM = 'wall + membrane' fraction.

A

 $M_r \times 10^{-3}$ 

B

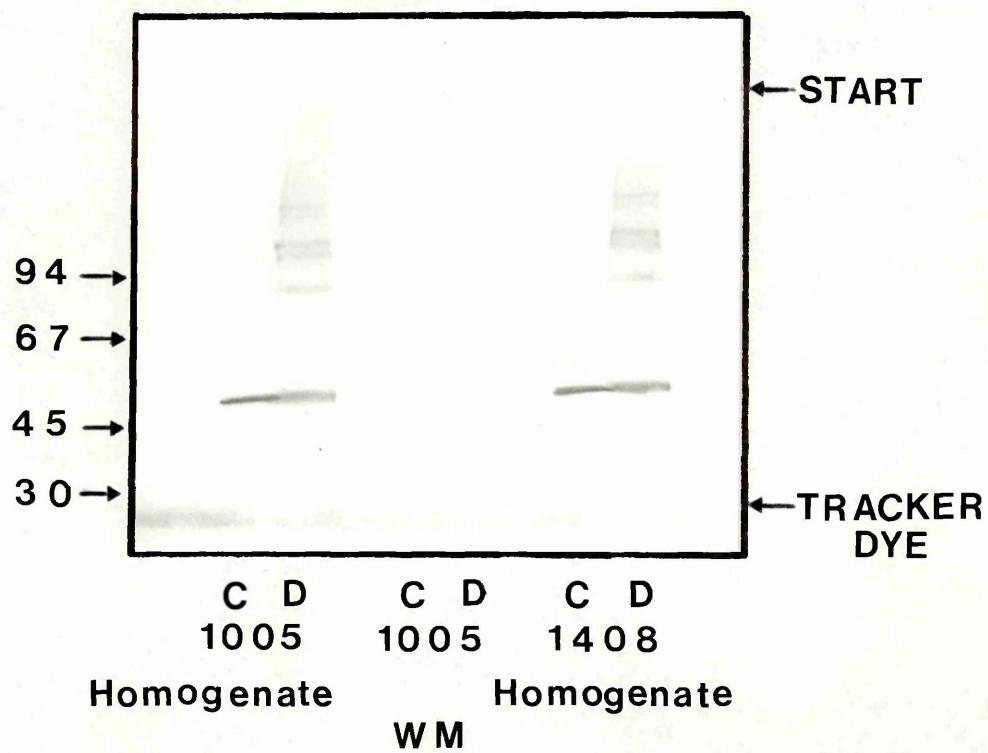
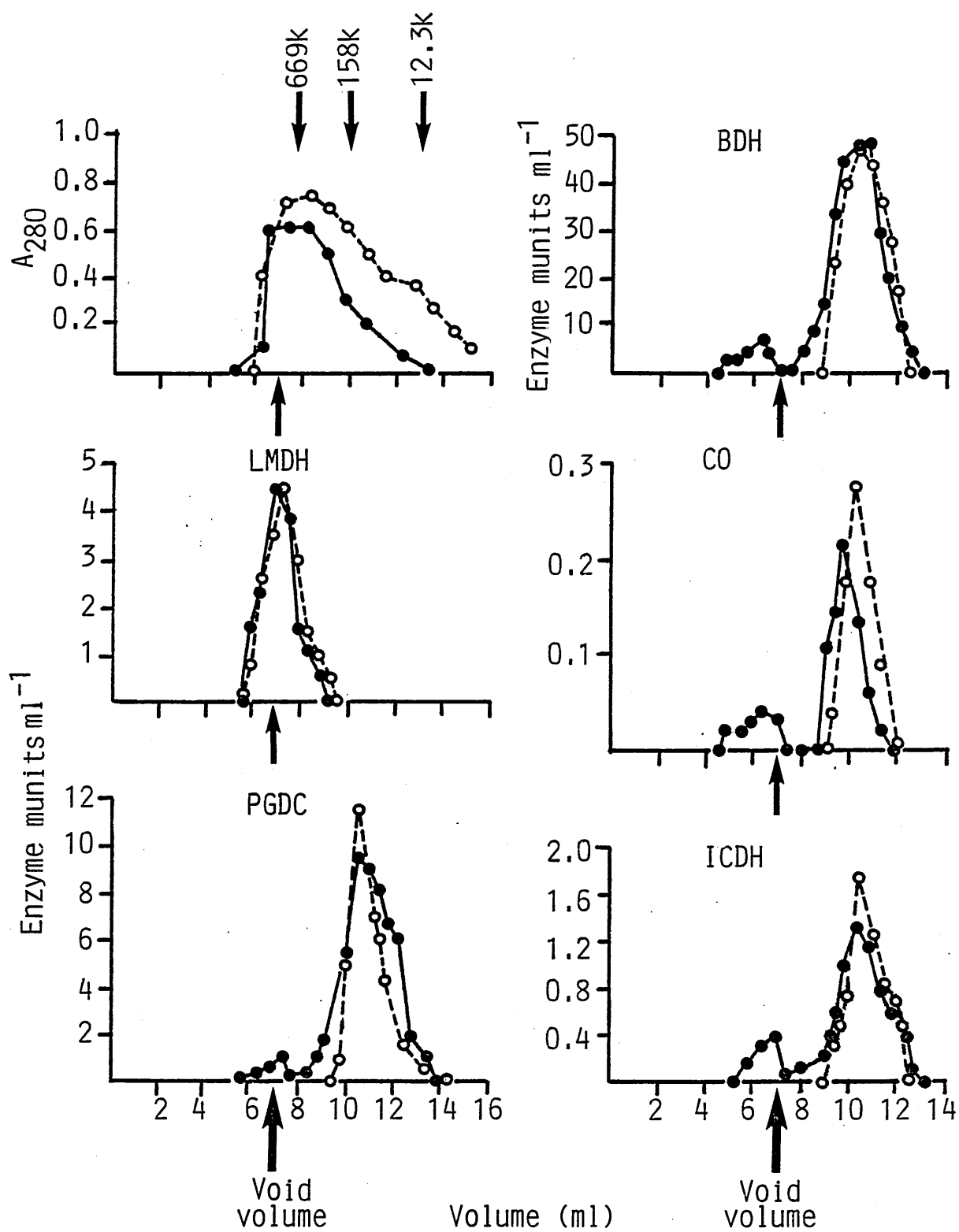


Figure 30 Gel filtration chromatography of control and DMSI extracts

Control (○---○) and DMSI (●—●) treated French press homogenates (Methods 2.14.4) from mutant strain C1005 (grown on complex media supplemented with D,L-mandelate) were applied to a gel filtration column (30cm long x 0.7cm diam.) of Ultrogel AcA 34 equilibrated with 0.1M potassium phosphate buffer, pH7.5 containing 100 μ M TPP, 100 μ M DTT, 2mM MgSO₄ and 1mM EDTA. Enzyme activity was eluted with the same buffer at a flow rate of 4ml h⁻¹ and fractions were collected every 7.5 min. The column was calibrated with proteins of known M_r values and the elution positions are indicated i.e. thyroglobulin, M_r = 669,000; aldolase, M_r = 158,000 and cytochrome c, M_r = 12,300. The elution volume of Blue Dextran (approx. M_r = 2,000,000) was taken as the void volume.



presence of 0.1M potassium phosphate buffer, pH7.5 containing 100 μ M TPP, 200 μ M DTT, 2mM MgSO₄ and 1mM EDTA) to immunoblotting with anti-LMDH and anti-PGDC. A similar pattern to that of the immunoblots of cross-linked French press homogenates from strain C1005 was obtained (results not shown).

3.5.14 Crosslinking intact bacteria and French press homogenates with PDM

The failure to crosslink LMDH and PGDC with the dimethylimidate crosslinkers could have been due to the absence of suitably positioned lysine residues on each protein and so the effect of PDM, which cross-links sulphhydryl groups, was studied.

Intact bacteria and French press homogenates from mutant strain C1005 were treated with PDM (Methods 2.15.1 and 2.15.2) after which extracts were immunoblotted with anti-LMDH and anti-PGDC. However, there was no evidence of species with higher M_r values even although PDM inhibited LMDH activity by approx. 40% (results not shown).

3.5.15 Immunoprecipitation of French press homogenates

French press homogenates from mutant strain C1005 and mutant strain C1219 were subjected to immunoprecipitation by anti-LMDH, anti-DMDH and anti-PGDC (Table 12). No LMDH activity was precipitated by anti-LMDH whereas only 27% DMDH and 0-5% PGDC remained in the supernatant after incubating extracts with anti-DMDH and anti-PGDC respectively. Neither anti-DMDH nor anti-PGDC had any effect on the other enzyme activities i.e. LMDH or PGDC in the case of anti-DMDH and DMDH or LMDH in the case of anti-PGDC.

Table 12 Immunoprecipitation of French press homogenates

Addition	% of starting enzyme activity					
	LMDH		DMDH		PGDC	
	C1005	C1219	C1005	C1219	C1005	C1219
Buffer	100	100	ND	100	100	100
Normal rabbit serum	96	91	ND	100	89	76
anti-LMDH	96	105	ND	115	113	105
anti-DMDH	96	105	ND	27	93	76
anti-PGDC	96	96	ND	108	ND	5.4

Bacteria (mutant strains C1005 and C1219 grown on complex media supplemented with D,L-mandelate) were disrupted by two passages through the French pressure cell (Methods 2.9.2) and the homogenates (100 μ l) were incubated with an equal volume of antisera (anti-LMDH, anti-DMDH or anti-PGDC) or with normal rabbit serum or buffer as controls for 60 min at 23°C. Protein A-Sepharose (100 μ l) was added and the samples were incubated for a further 60 min at 23°C before immunocomplexes were precipitated by centrifuging for 5 min in an Eppendorf 3200 centrifuge (Methods 2.28). The supernatants so formed were assayed for enzyme activities and the results are expressed as a percentage of the enzyme activities of the control (+ buffer) sample before centrifuging.

ND = not detectable.

CHAPTER 4 DISCUSSION

4.1 Separation of outer and inner membranes and location of the lactate and mandelate dehydrogenases of *A.calcoaceticus*

A method for disrupting *A.calcoaceticus* strain NCIB 8250 following lysozyme treatment was developed for several reasons. Firstly, lysozyme disruption of bacteria is generally regarded as a gentle method and minimises fragmentation of membranes (Mowbray & Moses, 1976; Scott et al., 1976) and so it was thought that this method would be useful in the study of enzyme interactions (3.5.3). Secondly, it was hoped that lysozyme treatment and subsequent separation of the outer and inner membranes could be used as a starting point for purifying LMDH. However, it became apparent that because of the time scale and large volumes that would have been required (during lysis of spheroplasts; see Methods 2.9.3.1) this method was inappropriate for the initial stages of the LMDH purification. Finally, inner and outer membrane separation was required for the location of the lactate and mandelate dehydrogenases.

That the outer and inner membranes of *A.calcoaceticus* were substantially separated was confirmed by enzyme distribution studies (Figure 5) as well as by analysing the membrane fractions by SDS PAGE (Figure 5) and electron microscopy (Figure 6). SDS PAGE of the outer membrane showed four major proteins (Figure 5) with M_r values of 44,000, 28,000, 22,000 and 13,000. This is similar to the banding pattern of the outer membrane of *P.aeruginosa* in which the four major outer membrane proteins have M_r values of 37,000, 35,000, 21,000 and 17,000 (Hancock & Nikaido, 1978). On the other hand, in *E.coli* (Schnaitman, 1970), *Salmonella typhimurium* (Osborn et al., 1972) and *Proteus mirabilis* (Hasin et al., 1975) there is one predominant outer membrane protein with an M_r of 44,000. The electron micrographs of the inner and outer membranes (Figure 6) were similar to those obtained by Scott et al. (1976) for *Acinetobacter* sp. strain H01-N and by Schnaitman (1970) for *E.coli*.

The lactate and mandelate dehydrogenases were principally located in the cytoplasmic membrane (Table 2) which is known to be involved in many cellular functions including selective permeation and active transport, electron transport, oxidative phosphorylation, and the biosynthesis of phospholipids, peptidoglycans and proteins (Salton, 1971).

4.2 Purification and characterisation of LMDH

The purification of integral membrane proteins is not an easy task and many workers have encountered difficulties relating to solubilising proteins which retain their biological activity and native structure while avoiding aggregation of proteins into non-physiological entities (Helenius & Simons, 1975; Tanford & Reynolds, 1976; Baines & Poole, 1985). The long standing problem of succinate dehydrogenase from E.coli is an example of such a protein system where conventional purification methods have been unsuccessful. However, structural information has been obtained indirectly using mutants (Spencer & Guest, 1974) and immunological methods (Owen & Candon, 1982) and more recently from the nucleotide sequence of the genes coding for succinate dehydrogenase (Wood et al., 1984; Darlison & Guest, 1984). The purification of LMDH (3.2) was not without problems and indeed the final purification was not entirely satisfactory because the yield was low and the final material was not completely homogeneous.

In the preliminary experiments on the purification, a three component system was observed on SDS PAGE and each component was present in almost equal amounts (Figure 7B). There were two possibilities for this: (1) unrelated proteins with similar solubility properties had been purified together or (2) the incorporation of these three polypeptides into a single micelle reflected a structural-functional relationship between these polypeptides in the intact membrane (Tanford & Reynolds, 1976) as is the case for succinate dehydrogenase (Hederstedt & Rutberg, 1981).

Tanford & Reynolds (1976) suggested that if detergent is present in excess, such that there are more detergent micelles than polypeptide chains, then detergent complexes containing one polypeptide chain per particle would predominate unless there are attractive forces that cause

two or more polypeptide chains to remain associated. Although it was possible to separate these three components (Figure 7B) in the purification scheme reported in Section 3.2.2, such that they were no longer present in equal ratios, it was not possible [even though several methods were studied (3.2.3.b)] to purify LMDH completely (Figure 13) and there was always some contamination by the polypeptides with M_r values of 66,000 and 27,000 in the final LMDH preparation. It may be that these two polypeptides are components of LMDH in its native environment. Evidence for this is that (1) LMDH is inactivated by high Triton X-100 concentrations especially during the initial solubilisation from the membrane (Allison *et al.*, 1985b), (2) the recovery of LMDH activity after ammonium sulphate precipitation (3.2.3.c) was variable (25-80%) probably because Triton X-100 was concentrated during the process and (3) the inability to completely purify LMDH (Figure 13). The inactivation of LMDH by high Triton X-100 concentrations may be due to the three components (Figure 7B) being separated into different micelles and indeed since the recovery of LMDH was very low (1.1%; Table 3), the activity in the final preparation may be due to the small amounts of the 66,000 and 27,000 polypeptides present (Figure 13).

One possible method for testing whether the polypeptides with M_r values of 66,000 and 27,000 are involved in LMDH activity would be to raise antibodies against these components to see if the antibodies could affect LMDH activity (or 'L-mandelate oxidase' activity in the membranes and other properties). However, this sort of experiment could only be interpreted if positive results were obtained since the antibodies may not affect the LMDH activity for other reasons (see below). Another possibility is that these two components (M_r 66,000 and 27,000) are structural elements important in anchoring LMDH in the membrane and not involved in LMDH activity per se.

Polyclonal antibodies were raised against LMDH by cutting a Coomassie Blue stained protein band from an SDS polyacrylamide gel (Methods 2.26, Figure 18). This antiserum showed a positive band on immunoblots of extracts containing LMDH activity which had been prepared from induced strain 8250 as well as constitutive mutant strains C1005 and C1219, whereas there was no band corresponding to LMDH in extracts from mutants C1123 and C1408 which contain no detectable LMDH activity (Figure 19). That no protein corresponding to LMDH was detected in extracts from C1123 and C1408 suggests that these mutants do not synthesise an inactive protein but that the LMDH gene has been silenced in some way or, less likely, that the mutated gene product is very labile or is not recognised by the antibodies.

Although the LMDH antiserum showed a positive band on immunoblotting (Figure 19), no enzyme inhibition by the antiserum could be demonstrated (3.3.10). So-called 'null' antibodies have been raised against other enzymes, such as lysozyme (Maron *et al.*, 1971; Arnon *et al.*, 1971), β -D-galactosidase (Frackelton & Rotman, 1980) and shikimate dehydrogenase (personal communication, Dr. I.D. Hamilton). Cinder (1977) has pointed out that as a rule, antisera contain predominantly non-inhibitory antibodies which do not interfere with enzyme catalysis. There are two types of antigenic determinants in proteins: (a) contiguous or linear determinants which are represented by a continuous stretch of amino acid residues found together in the primary structure of the protein and (b) discontinuous or conformational determinants which are formed by 3-dimensional folding of the protein, bringing together amino acid residues far apart in the protein's primary structure (Sela, 1969; Nagradava & Grozdova, 1978). Since the antibodies were raised against denatured LMDH (Methods 2.26) it is most likely that the anti-LMDH is reactive towards contiguous determinants which may be exposed only on denaturation of the enzyme. It should also be borne in mind that

adsorption of contaminants from the anti-LMDH was only possible after treatment with denatured non-induced strain 8250 extracts (Figure 19B). It is therefore not surprising that antibodies raised against epitopes exposed only after denaturation of the enzyme do not interfere with enzyme catalysis. The antibodies raised against DMDH and PGDC were, however, raised against the native enzymes (personal communication, Dr. I.D. Hamilton) and so it is more likely that these have been raised against discontinuous determinants; this is reflected in the ability of these antibodies to inhibit enzyme activity (3.3.10).

The partial characterisation of LMDH (3.3) allows us to make an overall comparison of the two mandelate dehydrogenases and the two lactate dehydrogenases (Table 13). The four dehydrogenases are similar in many respects and the striking similarities of the two D(-)-specific enzymes and the two L(+)-specific enzymes which were observed in crude extracts (Fewson & O'Donnell, 1981; Hills & Fewson, 1983a; Table 14) prevail in the purified enzymes (Tables 13 & 14). The properties summarised in Table 13 for the purified enzymes are largely unchanged from the properties of the enzymes in crude extracts (Fewson & O'Donnell, 1981; Hills & Fewson, 1983a, Table 14) which suggests that the four dehydrogenases have been purified in their native conformation. This is in contrast to some other membrane-bound dehydrogenases which have shown changes in pH optima, K_m values and substrate specificities on purification (e.g. Futai, 1973; Kohn & Kaback, 1973; Jones & Venables, 1983) which suggests that the native conformation has been altered during the purification of these enzymes.

The subunit M_r of LMDH is very similar to that of LLDH from A.calcoaceticus (Table 13) and to that of the membrane-bound LLDH from E.coli ($M_r = 43,000$; Futai & Kimura, 1977). The latter two enzymes behave as multimeric proteins on gel filtration analysis e.g. LLDH from A.calcoaceticus has an apparent M_r of 670,000 (Allison & Fewson, 1986);

Table 13. Properties of the four purified dehydrogenases

	D-Mandelate dehydrogenase	D-Lactate dehydrogenase	L-Mandelate dehydrogenase	L-Lactate dehydrogenase
Location	All are integral components of the cytoplasmic membrane			
Solubilisation (%) by non-ionic detergents (e.g. Triton X-100 or Lubrol PX)	87-91	83-88	60-70	55-76
Solubilisation (%) by ionic detergents (e.g. cholate or deoxycholate)	70-91	63-87	4-42	13-32
M _r (monomer)	59,000	63,000	44,000	40,000
pI value	5.5	5.8	4.2*	<4.0
pH optimum	8.0	7.7	7.5	7.5
Stereospecificity	All are stereospecific for their substrates			
K _m value (μM) for substrate	385 (In addition, D- lactate is a substrate but K _m =4.8mM)	308	186	83
Assay conditions	DCIP reduction measured in the presence of PMS or PES. All require BSA or Triton X-100 in the reaction mixture for full activity.			
Inhibitors	None is affected by a wide range of chelating agents. All are moderately susceptible to several thiol-blocking reagents. All are inhibited by oxalate.			
Sensitivity to <i>p</i> -chloromercuribenzoate (concn. giving 50% inhibition)	Very severe (<1μM)	Severe (approx. 5μM)	Moderate (approx. 30μM)	Severe (approx. 5μM)
Cofactor (non-covalently bound)	FAD	FAD	FMN	FMN

Data for this table were taken from section 3.3 and from Allison *et al.*, 1985 *a.* and *b.*; Allison and Fewson, 1986.

* Dr. N. Allison, personal communication.

Table 14 Comparison of the properties of purified LMDH with that of
LMDH in sonic extracts

	<u>Pure enzyme</u>	<u>Sonic extract</u>
K_m value (μM) for L-mandelate	186	207
pH optimum	7.5	7.8
Concentration (μM) of HgCl_2 giving 50% inhibition	1.0	3.0
Concentration (μM) of p-chloromercuri- benzoate giving 50% inhibition	30	200
% inhibition obtained with 20mM D-mandelate in the presence of 100 μM L-mandelate	60	69

Data for this table were taken from section 3.3 and Hills & Fewson (1983a)

however, the apparent M_r of LMDH as estimated by gel filtration is 64,000 (3.3.2) implying that the enzyme exists as a monomer which binds approx. 0.5mg Triton X-100/mg protein (calculated by subtraction). Purified DMDH and DLDH from A.calcoaceticus (Allison et al., 1985b) as well as the DLDH from E.coli (Futai, 1973; Kohn & Kaback, 1973) are also monomeric proteins. The apparent M_r of the purified LMDH obtained by gel filtration (64,000) is very different from the apparent M_r obtained at the gel filtration step during the purification (3.2.3 d; M_r = 300,000). Presumably the difference is due to more proteins being present in the Triton X-100 micelle in addition to LMDH at this earlier stage of the purification. Even although the elution pattern of standard molecular mass proteins is unaffected by the presence of Triton X-100 (Figure 10), the accuracy of molecular sizing of enzyme-Triton X-100 complexes is questionable (Tanford & Reynolds, 1976). There is also the possibility of erroneous subunit M_r estimations being made for integral membrane proteins on SDS PAGE (Helenius & Simons, 1975; Tanford & Reynolds, 1976; Campbell et al., 1984). This is due to integral membrane proteins binding more SDS (5-7g SDS/g protein) than a typical 'soluble' protein (1.4g SDS/g protein)(Helenius & Simons, 1975). The uncertainty involved in M_r estimations of integral membrane proteins means that comparisons and conclusions can only be made with some caution.

Purified DMDH was 80% inhibited by 2mM L-mandelate (Allison et al., 1985b); purified LMDH was also inhibited by the non-substrate enantiomer of mandelate but was much less sensitive to this inhibition in that LMDH was inhibited by 60% in the presence of 20mM D-mandelate. A similar inhibition of both DMDH and LMDH by the opposite isomer of mandelate was also demonstrated in crude extracts (Hills & Fewson, 1983a; Table 14).

Some NAD(P) independent lactate dehydrogenases from other bacteria have been shown to require divalent metals ions for activity and are

inhibited by metal-chelating agents (Olsen & Massey, 1979) but LMDH (Table 5) like DMDH, DLDH and LLDH (Allison et al., 1985b; Allison & Fewson, 1986) is not affected by metal ion chelators and therefore presumably has no metal ion requirement, unless the chelators are sterically prevented from removing the metal.

DMDH, DLDH and LLDH both in crude extracts (Hills & Fewson, 1983a; Fewson & O'Donnell, 1981) and the purified enzymes (Allison et al., 1985b; Allison & Fewson, 1986) are very sensitive to reagents which react with sulphydryl groups e.g. p-chloromercuribenzoate and mercuric chloride (Table 13). LMDH both in crude extracts and the purified enzyme is also inhibited by these reagents but is much less sensitive than the other enzymes (Tables 4, 13 & 14). This inhibition by thiol-blocking agents suggests that there may be reactive cysteine residues in the active sites of these dehydrogenases, although substrate-protection experiments followed by characterisation of active site peptides would be necessary to confirm this.

Although the difference spectrum of the purified LMDH (Figure 16) is different from the classical difference spectrum of 'soluble' flavoproteins [which usually have two peaks of approx. equal intensity at 380nm and 450nm (Mahler & Cordes, 1969)], the result obtained with LMDH (Figure 16) was indicative of it being a flavoprotein. This was confirmed when the fluorescence of the acid-extractable flavin from the purified LMDH was studied and identified as that of FMN (Figure 17). FMN has also been identified as the non-covalently bound prosthetic group of LLDH from A.calcoaceticus (Allison & Fewson, 1986) and of LLDH from E.coli (Futai & Kimura, 1977). By contrast, FAD was identified as the prosthetic group in both DMDH and DLDH from A.calcoaceticus (Allison et al., 1985b) and in this respect these latter two enzymes resemble the membrane-bound DLDH from E.coli (Futai, 1973) and the soluble DLDH from M.elsdenii (Brockman & Wood, 1975) both of which contain FAD.

The striking similarities of LMDH and LLDH and of DMDH and DLDH with reference to their subunit molecular masses, pH optima, susceptibility to sulphydryl group reagents and presence of FMN or FAD as cofactors (Table 13) may suggest that these enzymes have a common evolutionary origin. It is tempting to suggest that DMDH evolved as a result of a mutation in the gene encoding DLDH and similarly LMDH may have evolved from LLDH. However, DMDH and LMDH appear to be co-regulated (Hills & Fewson, 1983b) as do DLDH and LLDH (Allison et al., 1985a) and the genes responsible for DMDH and LMDH appear to map close to each other (Vakeria et al., 1984) and so it is possible that in mutants of A.calcoaceticus NCIB 8250, DMDH has arisen as a result of expression of a previously silent gene closely related to that coding for LMDH (Hills & Fewson, 1983b; Vakeria et al., 1984). At this stage, it is difficult to speculate on the evolutionary relationships of these four enzymes. Perhaps cloning and sequencing of the genes encoding these enzymes will elucidate their evolutionary origins.

4.3 Enzyme interactions in the mandelate pathway of *A.calcoaceticus*

The major part of this thesis involved a study of enzyme interactions in the mandelate pathway of *A.calcoaceticus*. Weak protein interactions are notoriously difficult to study because, by their very nature, they are easily disrupted. As a result of this easy disruption, experiments which show no evidence of enzyme interactions are inconclusive because it could be that enzyme associations do occur but that the techniques available are inadequate for their detection. Several approaches were therefore used in the study of the mandelate enzyme interactions in *A.calcoaceticus* (3.5). The aim of these experiments was to show whether or not the enzymes of the mandelate pathway interacted specifically. The physiological significance of such interactions (should they occur) was not studied.

4.3.1 Artifactual possibilities

Throughout this study the possibility that results were artifactual was always considered. Artifacts could arise as a result of non-specific binding in non-physiological buffers or trapping in vesicles. Many of the reported examples of 'soluble' enzyme interactions discussed in the Introduction (1.5.4; 1.5.5; particularly Mowbray & Moses, 1976; Gorringer & Moses, 1980) did not include the appropriate controls. In the present study, the behaviour of control enzymes was monitored; CO was taken as an example of a control 'soluble' enzyme from a related part of metabolism (Figure 1) whereas ICDH and GS were taken as examples of control 'soluble' enzymes from unrelated parts of metabolism. In addition, particular attention was paid to the pH and ionic strength of buffers used (Table 9) and frozen bacteria and extracts were not used since freezing and thawing would probably disrupt any protein interactions. Several workers have emphasised the need to work under physiological conditions of protein concentration, pH and ionic strength (Clarke & Masters, 1975; Clegg, 1984b); no doubt this is fundamentally correct but Clegg (1984a)

is of the opinion that we simply do not know enough about cellular conditions and therefore cannot mimic them. Fulton (1982) and Srere (1980) have gone as far as to say that the cytoplasm does not exist as an aqueous solution since the protein concentration is so high (approx. 50%) and that protein crystals (approx. 40% of which are water) would be useful models for considering conditions in the cell.

4.3.2 Evidence for co-purification

If enzymes associate, it should be possible to co-purify them, and so several methods were used to investigate the possible co-purification of the mandelate enzymes.

a) Ultracentrifugation

Extracts prepared after disrupting bacteria by sonication, French pressing and lysozyme treatment were examined by ultracentrifugation (3.5.3; Figures 22 & 23). Sonication is a rather harsh method of disruption and therefore probably results in the membranes being broken into small fragments which do not sediment. This would account for the observation that only 50-60% LMDH was recovered in the pellet after sonication (Figure 22A) whereas approx. 95% LMDH sedimented after lysozyme treatment (Figure 22B) and French press disruption (Figure 23). PGDC is a rather large enzyme with an apparent M_r of 230,000 (Barrowman & Fewson, 1985) and so there was the possibility that some PGDC would sediment because of the high M_r of this enzyme. GS was therefore assayed to illustrate the behaviour of a very large 'soluble' enzyme (M_r = 600,000) under the conditions of centrifugation used; 30-40% GS sedimented irrespective of the method of disruption (Figures 22 & 23) whereas the amount of PGDC recovered in the pellet varied (5-55%) with the disruption conditions (Figures 22 & 23) indicating that the presence of PGDC in the pellet was not a result of its high M_r . The indirect assay for PGDC involves linking PGDC activity to that of alcohol dehydrogenase (Figure 20) and so the 40-50% PGDC recovered in the pellet after 1 or 2 passages

through the French press is probably not trapped in vesicles since alcohol dehydrogenase would not have access to such trapped PGDC.

Asakawa et al. (1968) reported a similar variation in the distribution of phenylpyruvate decarboxylase from Archromobacter eurydice [Brisou & Prevot (1954) suggested that this bacteria should be classified as A.calcoaceticus] which had been disrupted in different ways. When extracts were prepared by grinding bacteria with quartz sand, 95% of the phenylpyruvate decarboxylase was recovered in the high speed supernatant consistent with this being a 'soluble' enzyme whereas, surprisingly, when the bacteria were disrupted by sonication, 20-25% of the enzyme was located in the high speed pellet (Asakawa et al., 1968). Phenylpyruvate decarboxylase and PGDC from A.calcoaceticus are very similar enzymes (Barrowman & Fewson, 1985) and so this property of differential sedimentation may be an inherent property of these enzymes or it may reflect a specific association with the membrane fraction.

It is well established that the orientation of membrane vesicles varies with the method of disrupting bacteria. Disruption by sonication or French pressing results in inverted (inside out) membrane vesicles (Rosen & McClees, 1974; Futai, 1974; Yamato et al., 1978) whereas disruption by lysozyme produces right-side-out vesicles (Yamato et al., 1978). There was no evidence of PGDC specifically interacting with the membrane fraction after lysozyme treatment (Figure 22B), however, owing to the membrane vesicles being right-side-out after lysozyme treatment (Yamato et al., 1978), there is the possibility that PGDC interacts with the inner side of the membrane and is therefore trapped inside these vesicles and is inaccessible to assay.

Not only did the amount of PGDC associated with the membrane vary with different methods of disrupting bacteria (Figures 22 & 23) but the protein concentration of the French press homogenate had a dramatic effect on the amount of PGDC recovered in the pellet (Figure 24). Although there are apparently no data for prokaryotic intracellular protein concentrations, the protein concentration in eukaryotic cytoplasm

and in mitochondria is said to be as high as 50% (Sols & Marco, 1970; Ottaway & Mowbray, 1977; Srere, 1981, 1982, 1984) and so perhaps this protein concentration dependent interaction of PGDC with the membrane takes place in vivo.

High speed pellets were washed by different conditions in an effort to elucidate the interaction of PGDC with the membrane (3.5.6). Conditions commonly used to dissociate extrinsic membrane proteins (Tables 10 & 11) were used to try to achieve a differential release of ICDH while leaving PGDC associated. In all cases, where ICDH was washed off, PGDC was also washed from the pellets; however, there was always more PGDC than ICDH recovered in the membrane fraction. The difficulty in this study was in trying to maintain a balance between keeping the protein concentration high and minimising non-specific binding. All other attempts to wash non-specifically bound protein from the membrane proved to be unsuccessful since a condition was not found where ICDH could be washed from the membrane while leaving PGDC associated. Of course, this could mean that PGDC does not specifically interact or it may be that any specific interaction was disrupted during the handling of samples.

b) Gel filtration chromatography

Analysis of French press homogenates or high speed pellets by gel filtration chromatography gave no evidence of co-elution of LMDH and PGDC (Figure 26). This could possibly have been due to dilution of the sample during the chromatography resulting in the dissociation of the enzymes. Weitzman & Barnes (1985) and Barnes & Weitzman (1986) found it necessary to include either 20% (v/v) glycerol, 14% (w/v) PEG or BSA (10mg ml^{-1}) in all buffers used in the gel filtration study of the Krebs cycle enzyme interaction in E.coli. However, PEG was shown to interfere with the association of PGDC with the membrane (3.5.9) and so was not included in the present gel filtration experiments. In contrast to the results reported by Barnes & Weitzman (1986) for the Krebs cycle enzymes,

there was no evidence for the co-elution of LMDH and PGDC even in the presence of 20% (v/v) glycerol or high protein concentrations (30mg ml^{-1}) (3.5.8).

c) Density gradient centrifugation : Percoll gradients

French press homogenates were analysed by density gradient centrifugation on Percoll gradients (Figure 25) which have proved useful in the studies of glycosomes (Oppendoes, 1981) and of plasma membranes (Belsham *et al.*, 1980). The advantage of these gradients is that their running time (approx. 30 min) makes them far more convenient than other density gradients (e.g. sucrose) whose extended running conditions (24-48h) may have possible detrimental effects on weak interactions. However, there was no evidence of co-sedimentation of LMDH and PGDC on Percoll gradients (Figure 25), even in the presence of high protein concentrations.

4.3.3 Immunoprecipitation

It was thought that immunoprecipitation could be a useful technique for studying enzyme interactions in that an antibody raised against one enzyme could co-precipitate an associating enzyme. Such an effect was observed in a study on the pyruvate dehydrogenase complex (De Marcucci, 1985). It was possible to immunoprecipitate lipoamide dehydrogenase (E3) from bovine kidney cells using antisera raised against pyruvate dehydrogenase complex even although this antisera did not cross-react with E3 on immunoblotting the purified complex. On the other hand, E3 was not immunoprecipitated from pig kidney cells and this was taken to reflect a difference in the association of E3 with the pyruvate dehydrogenase complex in these two cell lines (De Marcucci, 1985). However, under the conditions of immunoprecipitation used, there was no evidence of the antibodies raised against DMDH, LMDH or PGDC having any effect on the other enzyme activities (Table 12).

4.3.4 Use of crosslinkers

Chemical crosslinkers have been used with great success in the study of subunit structures of oligomeric proteins (Davis & Stark, 1970; Coggins, 1978). More recently, these reagents have been used to study 'nearest neighbours' of mitochondrial enzymes in situ (D'Souza & Srere, 1983) and of ribosomal proteins (Maassen, 1979) as well as in studies on pyruvate dehydrogenase (Packman & Perham, 1982; Packman et al., 1982) and the enzymes in the glycosome of T.brucei (Aman et al., 1985). Of more relevance to the present study, Halpin et al. (1981) reported in vivo stabilisation of a putative complex of the mandelate enzymes from P.putida using the crosslinking reagent DMSI.

When the experiments conducted by Halpin et al. (1981) on P.putida were repeated (Table 6), their results were confirmed in that there was a 5-50 fold increase in the specific activities of the 'soluble' mandelate enzymes recovered in the high speed pellet after DMSI treatment. However, this was not a specific effect since there was not only an increase in the mandelate enzymes but also an increase in the control enzymes CO, ICDH and GS (Table 6). Halpin et al. (1981) reported a similar increase in the specific activities of CO and GS in the pellets after DMSI treatment (Table 7) but did not interpret this as indicating that non-specific crosslinking had taken place. Unfortunately, Halpin et al. (1981) did not express their results as a percentage of total enzyme activity but in fact only 2-9% of the 'soluble' enzymes was found in the high speed pellet whereas approx. 95% of ICDH was recovered in this fraction (Table 6).

On analysis of control and crosslinked complexes on gel-filtration chromatography, Halpin et al. (1981) suggested that there was an 'all or nothing' trapping of the high molecular weight complex of proteins since there was little evidence of dimers, trimers or tetramers of the individual enzymes and so concluded that the crosslinking was not

completely random. However, when similar gel filtration experiments were carried out on Ultrogel AcA 34 which has a higher resolving power than Ultrogel AcA 22 which was used by Halpin *et al.* (1981), it could be seen that a substantial amount of non-specific crosslinking had taken place (Figure 21).

The results obtained with *in vivo* crosslinking of A.calcoaceticus (Table 8) were similar to those for P.putida (Table 6) in that only non-specific crosslinking was observed. It was thought that a specific effect could have been obtained if the bacteria were supplied with substrates and cofactors for the mandelate enzymes since if a transient complex exists it may only form in the presence of these factors. In order that the substrates and cofactors could enter the bacteria, they were treated with toluene in the presence of PEG (3.5.2). Toluene permeabilises the membrane (Beggs & Fewson, 1977) whereas PEG is thought to prevent cytoplasmic proteins leaking from the bacteria after permeabilisation (Matlib *et al.*, 1977). Slightly more crosslinking was observed after toluene/PEG treatment and crosslinking was obtained in stationary phase bacteria (3.5.2), suggesting that the crosslinker cannot normally enter stationary phase bacteria. However, no specific crosslinking was achieved in the presence of substrates and cofactors for the mandelate enzymes (3.5.2).

The inability to demonstrate specific crosslinking between LMDH and PGDC in these *in vivo* experiments (3.5.2) may have been because TNM buffer, pH8.0 had been used [to allow a direct comparison with the results reported by Halpin *et al.* (1981)]. Since the PGDC tetramer from A.calcoaceticus is known to dissociate at this pH (Barrowman, 1982) there is the possibility that the buffer conditions used (TNM buffer, pH8.0) are detrimental to any enzyme interactions which may occur. Furthermore, differential centrifugation may be an inadequate method for detecting crosslinking especially since only 60% LMDH was recovered in

the pellet fraction in these experiments (Table 8) and so there is the possibility that PGDC had been crosslinked to this 'soluble' species of LMDH. It is generally thought that a high pH (≥ 8.0) is required for the activity of the dimethylimidate crosslinkers. However, a method was developed which allowed crosslinking with DMSI and DMPI at pH 7.5 (Figure 27). Furthermore, control and crosslinked extracts were analysed by immunoblotting with antibodies raised against DMDH, LMDH and PGDC (3.5.10). Although some non-specific crosslinking was expected (on the basis of the earlier in vivo experiments, 3.5.2) it was anticipated that any significant specific crosslinking would be detected by immunoblotting.

In the first instance these experiments were carried out on crosslinked French press homogenates (Figure 28) but similar results were obtained after analysing extracts prepared after in vivo crosslinking at pH 7.5 by immunoblotting (3.5.13). The results obtained (Figure 28) confirmed that the crosslinking reaction had taken place since high M_r species were detected with anti-LMDH, anti-PGDC and anti-DMDH although in the latter case, it appeared that DMDH had been crosslinked to a variety of species with different M_r values. There was also evidence of intra-molecular crosslinking of DMDH (Figure 28). In addition to others, there was a band with an apparent M_r of 100,000 and one of 98,000 in the immunoblots obtained with anti-LMDH and anti-PGDC respectively (Figure 28). If LMDH and PGDC associate, one would expect to see a band with an apparent M_r of 102,000 [by addition of the M_r of LMDH (44,000) and that of PGDC (58,000) and assuming that crosslinked proteins do not behave differently from uncrosslinked proteins on SDS PAGE] on both the blots obtained with anti-LMDH and anti-PGDC. To try to establish whether the band with an apparent M_r of approx. 100,000 was a crosslinked species of LMDH and PGDC or due to crosslinking of both enzymes to some other unknown species, a sample with no LMDH

activity but with PGDC activity and conversely, one with LMDH activity but no PGDC activity were crosslinked (Figure 29). The same pattern was obtained as before (Figure 28), in that anti-LMDH reacted with a protein with an apparent M_r of 100,000 and anti-PGDC gave a positive reaction with a species with an apparent M_r of 98,000 whether or not PGDC or LMDH activity, respectively, was present (Figure 29) and so it can be concluded that LMDH and PGDC were crosslinked to some other cellular component but not to each other. The non-specificity of crosslinking with DMSI was also confirmed by analysing control and DMSI treated French press homogenates by gel filtration chromatography (Figure 30).

The dimethylimidate crosslinking reagents react with lysine residues on proteins and crosslinking between adjacent proteins takes place if there are two lysine residues, one on each protein, situated a fixed distance apart (dictated by the length of the crosslinking reagent)(Coggins, 1978). Even although several dimethylimidates of different lengths were studied (3.5.2), no specific crosslinking between LMDH and PGDC was observed and this may have been because there were no lysine residues in the correct positions. The effect of PDM, a bifunctional reagent which reacts with sulphhydryl groups (Reisler et al., 1974), was studied in the hope that there would be two suitably positioned sulphhydryl groups on LMDH and PGDC (3.5.14). However, there was no evidence of specific crosslinking of LMDH and PGDC when either intact bacteria or French press homogenates were treated with PDM (3.5.14).

4.3.5 Conclusions

This study (3.5) illustrates the difficulties in investigating weak protein interactions. Although several approaches have been used to study enzyme interactions in the mandelate pathway in A.calcoaceticus, there is only one positive piece of evidence (Figures 23 & 24) which indicates that PGDC interacts with the membrane fraction. It is not possible to say with certainty that LMDH and PGDC do not specifically

interact because the trouble with negative results in these studies is that there is always the possibility that the correct conditions had not been found or that the techniques available are inadequate for demonstrating weak protein interactions. The results obtained on differential centrifugation of French press homogenates (Figures 23 & 24) may be due to an inherent 'stickiness' of the PGDC molecule which is accentuated at high protein concentrations. There is also the possibility that PGDC interacts specifically with another unknown membrane component and that LMDH and PGDC are linked via this unknown species.

4.3.6 Future experiments

Two major questions remain to be answered:

1) Do enzyme interactions take place in prokaryotes?

Since there is the possibility that enzyme interactions are disrupted on breaking bacteria it may not be possible to isolate such complexes and so elucidation of enzyme interactions may have to rely on indirect approaches including studies using purified enzymes (Srere, 1985). However, there are many difficulties in translating from the binding properties of purified enzymes in vitro to the realities and complexities in the cellular environment. This notwithstanding, evidence for the specificity of enzyme interactions may be achieved using purified enzymes either in solutions which mimic cellular conditions e.g. PEG mimics high protein concentrations (Fahien & Kmoteck, 1979; Sumegi et al., 1985; Datta et al., 1985) or immobilised on Sepharose columns either directly or via antibodies (Koch-Schmidt et al., 1977; Mosbach, 1978; Mosbach & Mattiasson, 1978). Such studies have not been carried out on the mandelate enzymes because of the low yields of the purification procedures it has not yet been possible to purify sufficient amounts of DMDH, LMDH or PGDC (Section 3.2; Barrowman & Fewson, 1985; Allison et al., 1985b). Perhaps after the mandelate genes have been cloned and overexpressed and the mandelate dehydrogenases and PGDC have been

purified in much greater quantities, it may be possible to determine whether these enzymes interact specifically or not. In addition, binding studies with purified PGDC and inner membranes could be carried out.

- 2) If enzyme interactions take place in the cell, what advantages, if any, do they confer on the cell?

Some of the possible advantages of enzyme interactions discussed in the Introduction (1.5.2) may be illustrated using purified enzymes co-precipitated in PEG solutions (Datta et al., 1985) or co-immobilised on inert matrices (Srere et al., 1973; Koch-Schmidt et al., 1977); e.g. Datta et al. (1985) have demonstrated substrate channeling of oxalacetate in a complex of malate dehydrogenase and citrate synthase which had been precipitated from a PEG solution. In addition, mutants may prove useful in these studies, e.g. if a mutated protein is unable to interact with the next enzyme of a pathway then such a mutant would not experience the possible advantages of enzyme interactions (1.5.2).

Owing to the possibility that conditions used in experiments on purified enzymes may be so far removed from cellular conditions that such studies are of no physiological significance, a better understanding of metabolic compartmentation could possibly be obtained from in vivo isotope tracer experiments (Shipley & Clark, 1972; Srere & Mosbach, 1974; Ottaway & Mowbray, 1977; Bharagava, 1985; Bernhard & Srivastava, 1986). The disadvantages relating to difficulties in the interpretation of such experiments discussed in the Introduction (1.5.2b) may partly be overcome by the development of high resolution techniques, such as HPLC, and by the increased sensitivity of techniques such as NMR, for the detection of labelled intermediates. In addition, a thorough knowledge of the effectors controlling the individual enzymes and their effect on the flux through the system under study would be required for any meaningful interpretation of data from isotope tracer experiments. Perhaps it will

only be worth studying the possibility of enzyme interactions in systems where substrate channeling can be demonstrated.

5. References

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